FIGURE 1

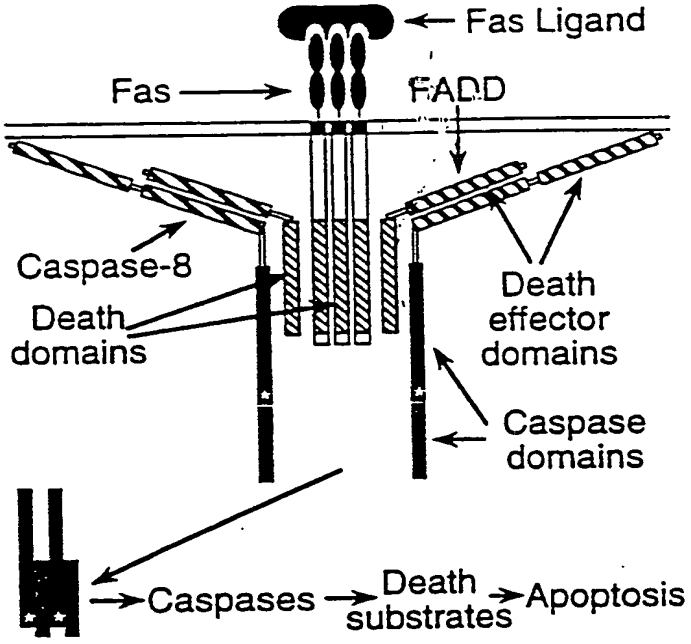


FIGURE 2

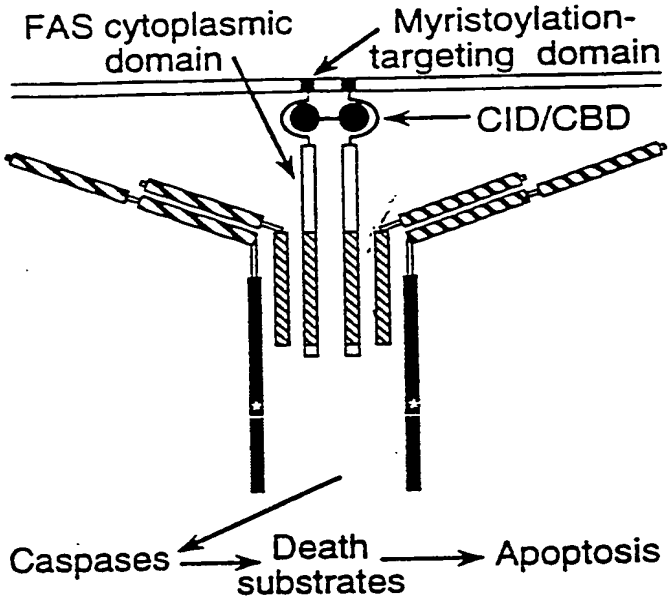


FIGURE 3

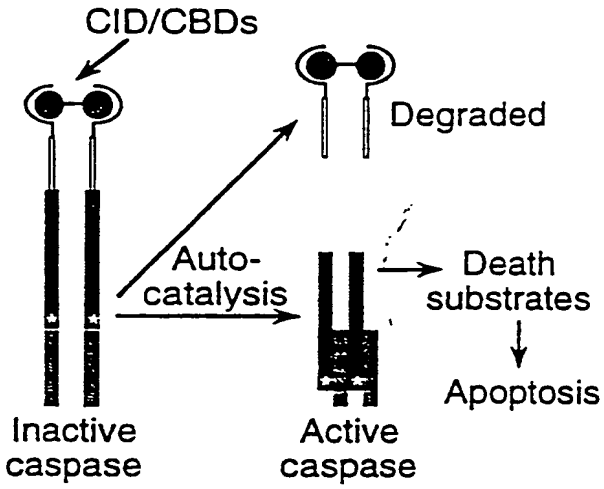


FIGURE 4

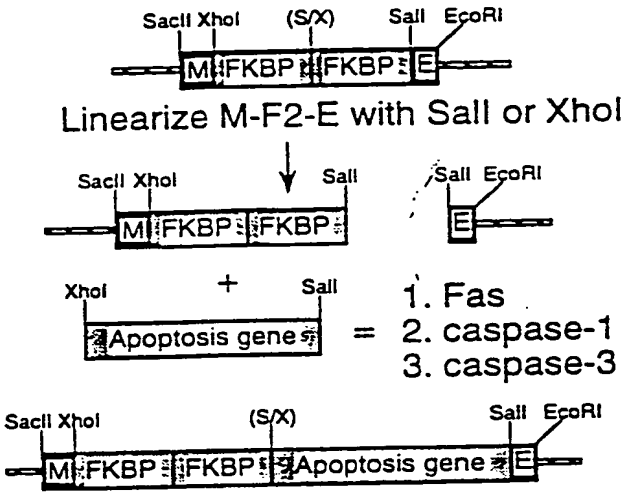

SacII XhoI (S/X) SalI EcoRI
M FKBP FKBP E
Linearize M-F2-E with SalI or XhoI
SacII XhoI SalI
SalI EcoRI
M FKBP FKBP
E
XhoI + SalI
Apoptosis gene
= 1. Fas
2. caspase-1
3. caspase-3
SacII XhoI (S/X) SalI EcoRI
M FKBP FKBP Apoptosis gene E

FIGURE 5

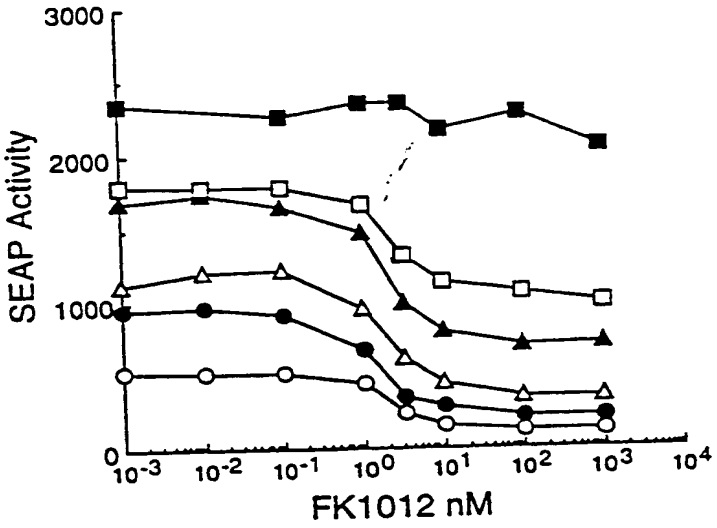

3000
2000
1000
0
SEAP Activity
10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4}
FK1012 nM

FIGURE 6

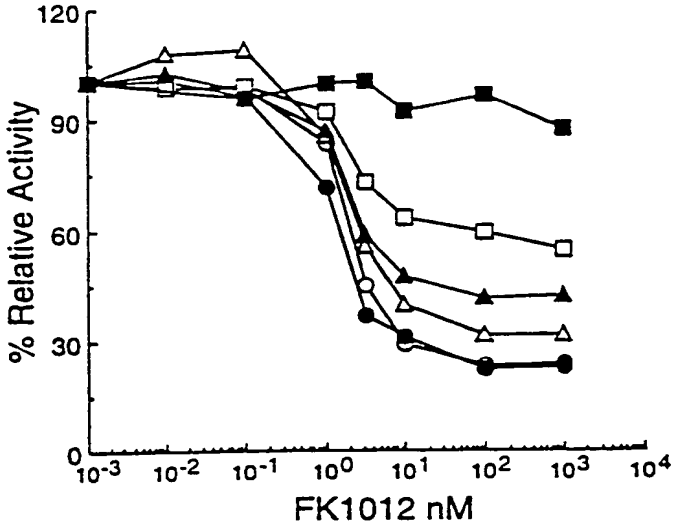

120
90
60
30
0
% Relative Activity
10^{-3} 10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4}
FK1012 nM

FIGURE 7

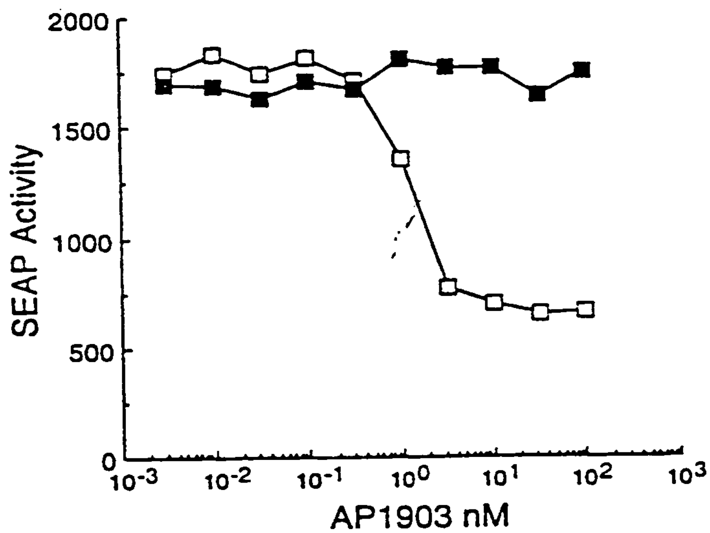


FIGURE 8

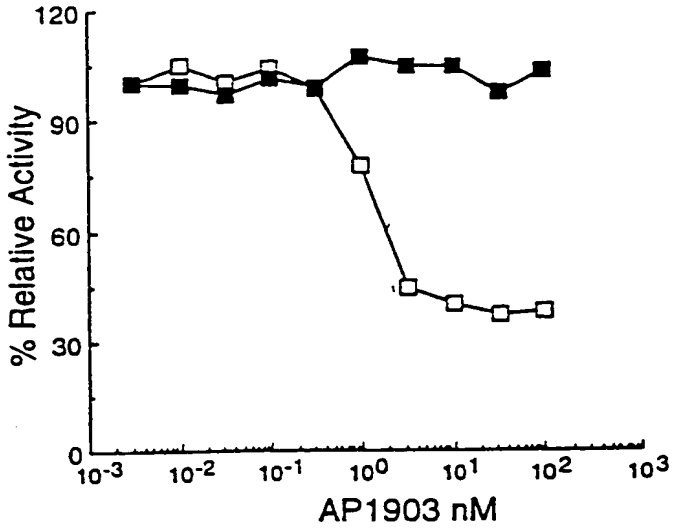

120
90
60
30
0
% Relative Activity
10-3
10-2
10-1
100
101
102
103
AP1903 nM

FIGURE 9

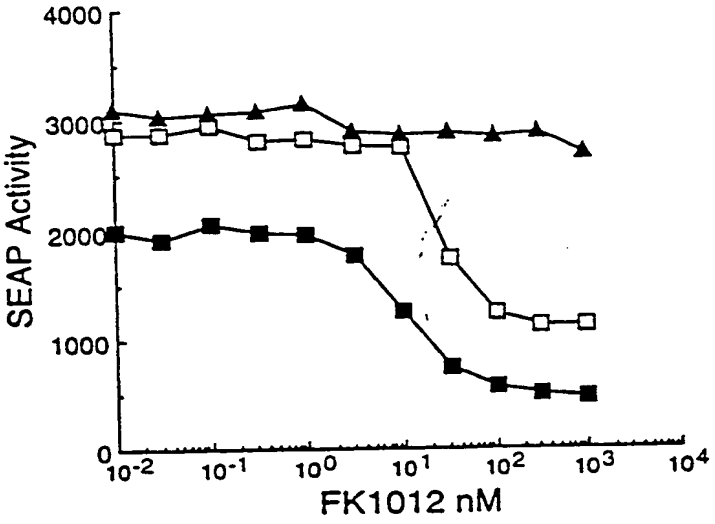

4000
3000
2000
1000
0
SEAP Activity
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
FK1012 nM

FIGURE 10

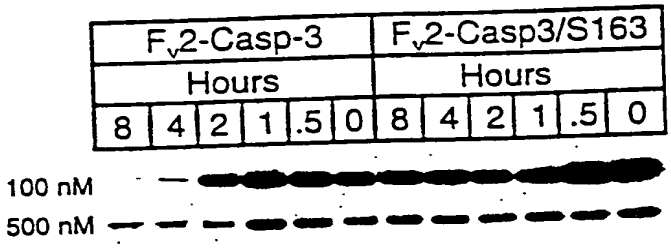


FIGURE 11

F_v2-Casp-3						F_v2-Casp3/S163					
AP1903 nM						AP1903 nM					
100	32	10	3.2	1	0	100	32	10	3.2	1	0

FIGURE 12

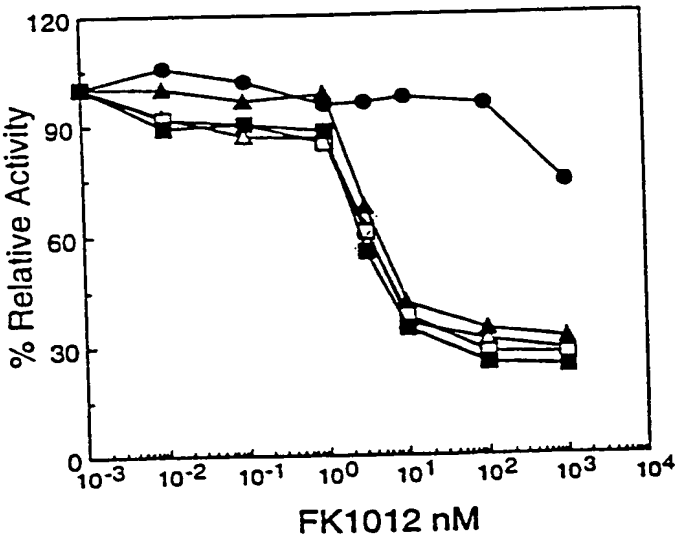

120
90
60
30
0
% Relative Activity
10-3
10-2
10-1
100
101
102
103
104
FK1012 nM

FIGURE 13

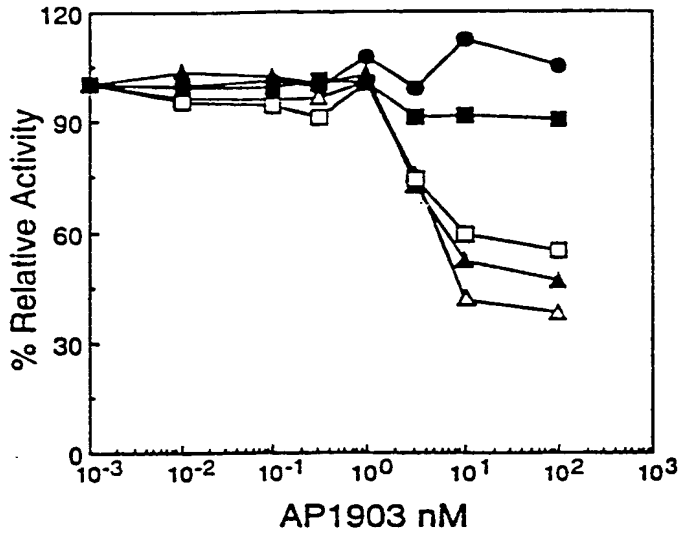

120
90
60
30
0
% Relative Activity
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
AP1903 nM

FIGURE 14

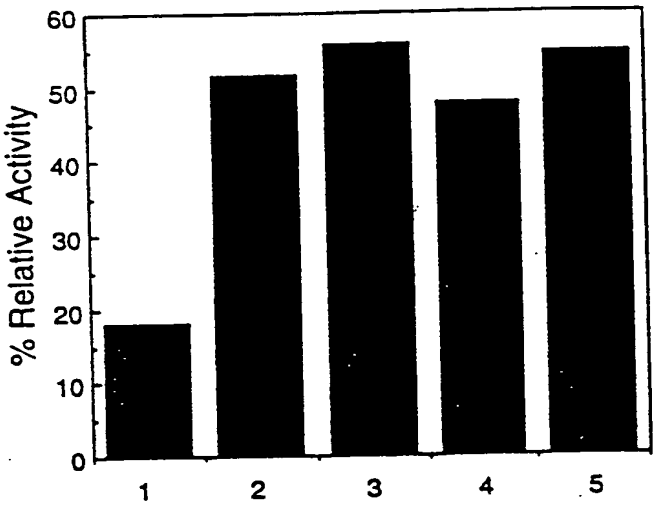


FIGURE 15

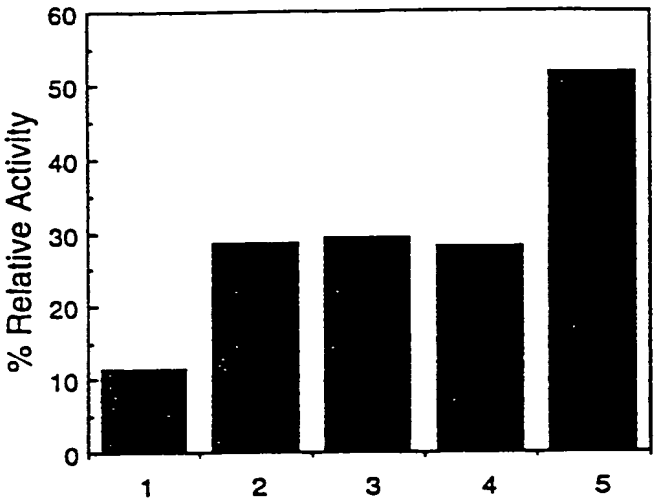


FIGURE 16

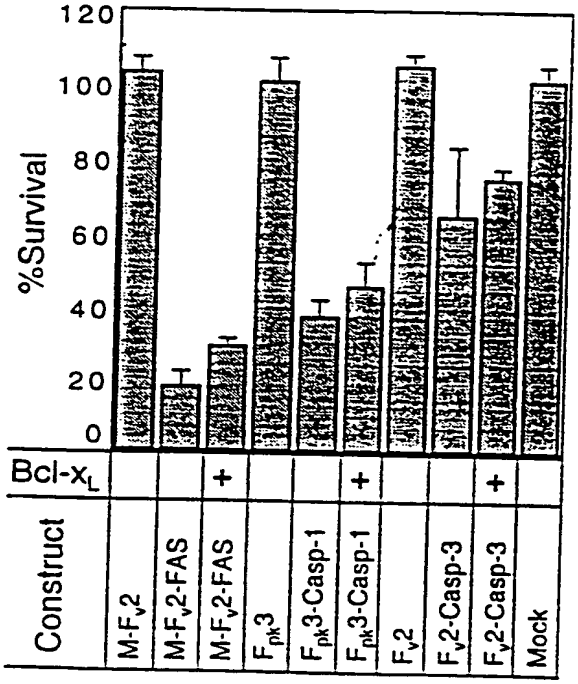

120
100
80
60
40
20
0
%Survival
Bcl-x_L
Construct
M-F_v2
M-F_v2-FAS
M-F_v2-FAS
F_{nk}3
F_{nk}3-Casp-1
F_{nk}3-Casp-1
F_v2
F_v2-Casp-3
F_v2-Casp-3
Mock
+
+
+

FIGURE 17

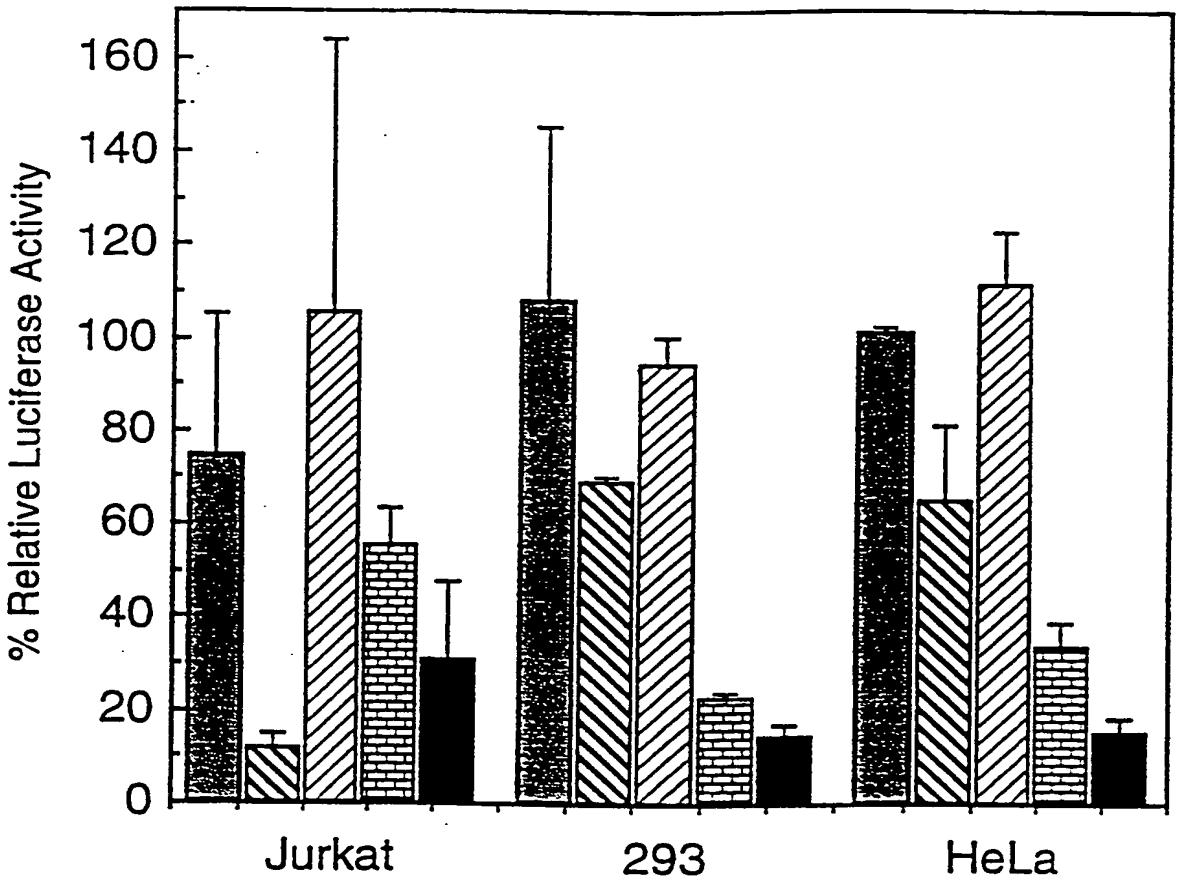
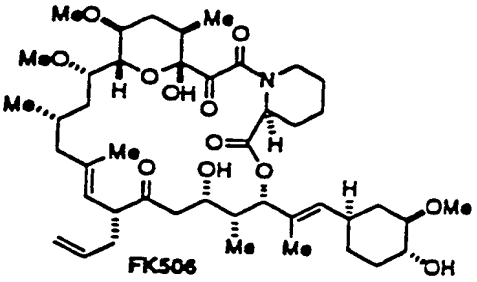

% Relative Luciferase Activity
160
140
120
100
80
60
40
20
0
Jurkat
293
HeLa

FIGURE 18



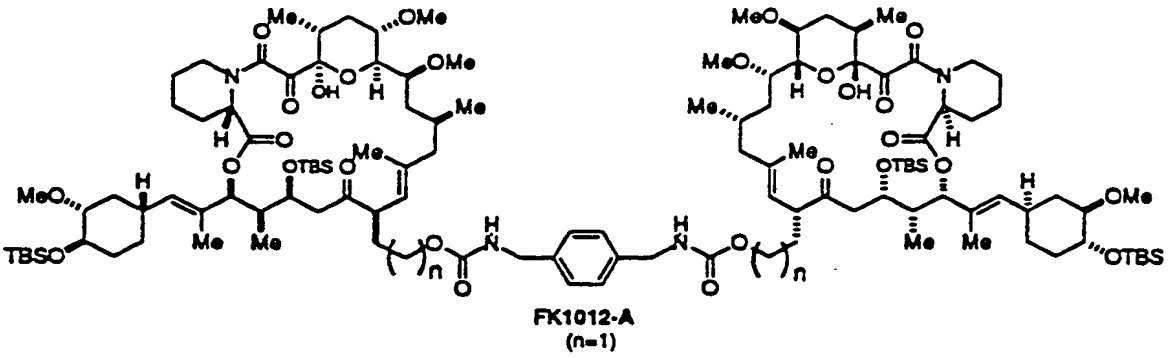
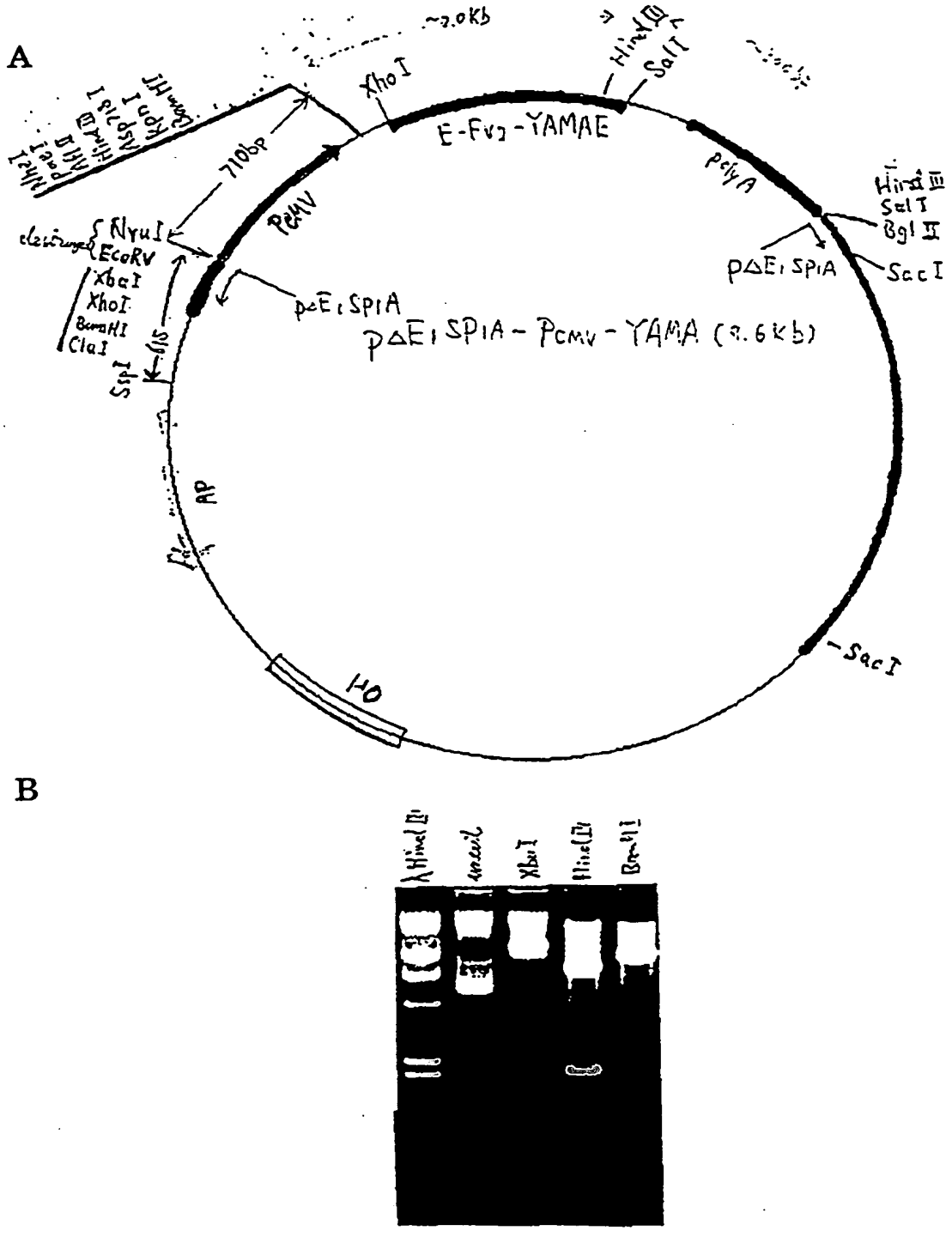


FIGURE 19

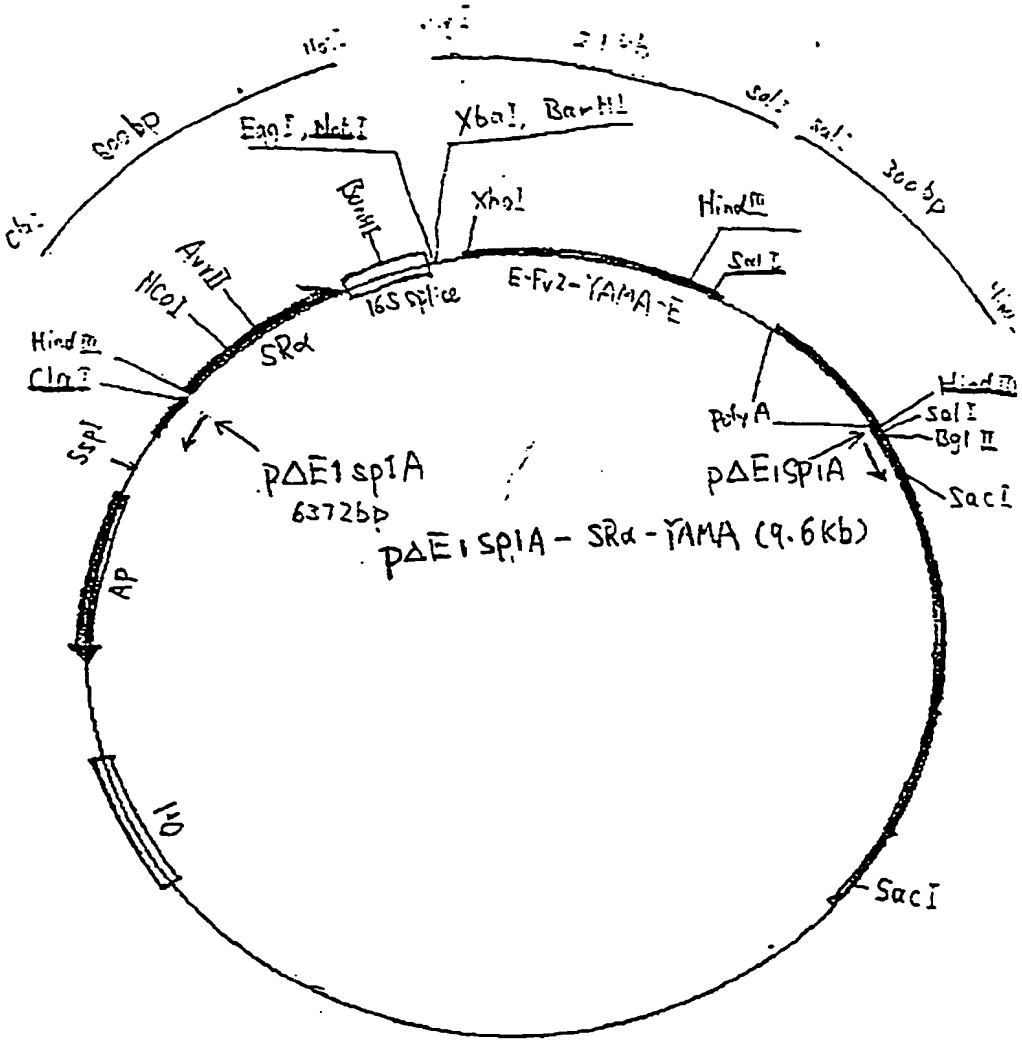

A
~3.0Kb
XhoI
HindIII
SalI
E-Fv1-YAMAE
polyA
HindIII
SalI
BglII
SacI
pΔE1SP1A
710bp
PCMV
NheI
PacI
AflII
NruI
HindIII
Asp718I
KpnI
BamHI
NruI
EcoRV
XbaI
XhoI
BamHI
ClaI
SpeI
SpeI
pE1SP1A
pΔE1SP1A-PCMV-YAMA (3.6Kb)
AP
Ori
IHO
SacI

B

λ HindIII
uncut
XbaI
HindIII
BamHI

FIGURE 20

A


EagI, NotI
XbaI, BamHI
BamHI
XhoI
HindIII
SalI
AvrII
NCoI
16S splice
E-Fv2-YAMA-E
HindIII
ClaI
SRα
PolyA
HindIII
SalI
BglII
SacI
SpeI
pΔE1spIA
63726p
pΔE1spIA
pΔE1spIA - SRα - YAMA (9.6kb)
AP
ori
SacI

B

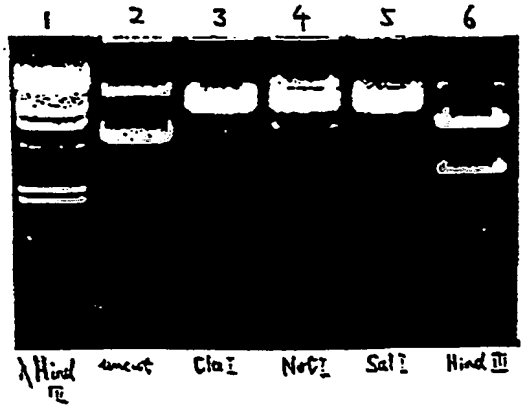

1
2
3
4
5
6
λHindIII
uncut
ClaI
NotI
SalI
HindIII

FIGURE 21

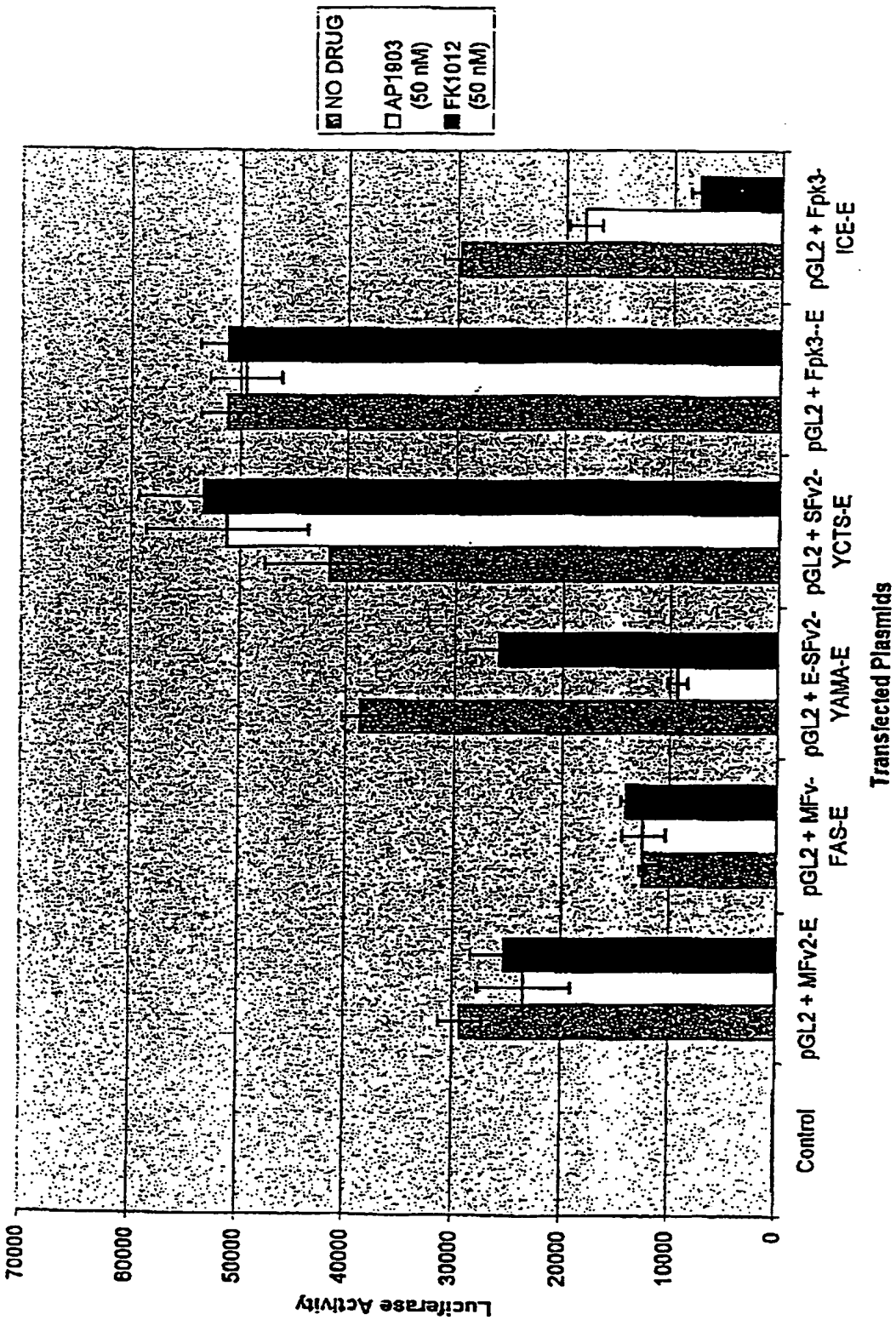

CID-Mediated Apoptosis In BPH-derived CR-2a SMC
NO DRUG
AP1903 (50 nM)
FK1012 (50 nM)
Luciferase Activity
70000
60000
50000
40000
30000
20000
10000
0
Control
pGL2 + MFv2-E
pGL2 + MFv-FAS-E
pGL2 + E-SFv2-YAMA-E
pGL2 + SFv2-YCTS-E
pGL2 + Fpk3-E
pGL2 + Fpk3-ICE-E
Transfected Plasmids

FIGURE 22

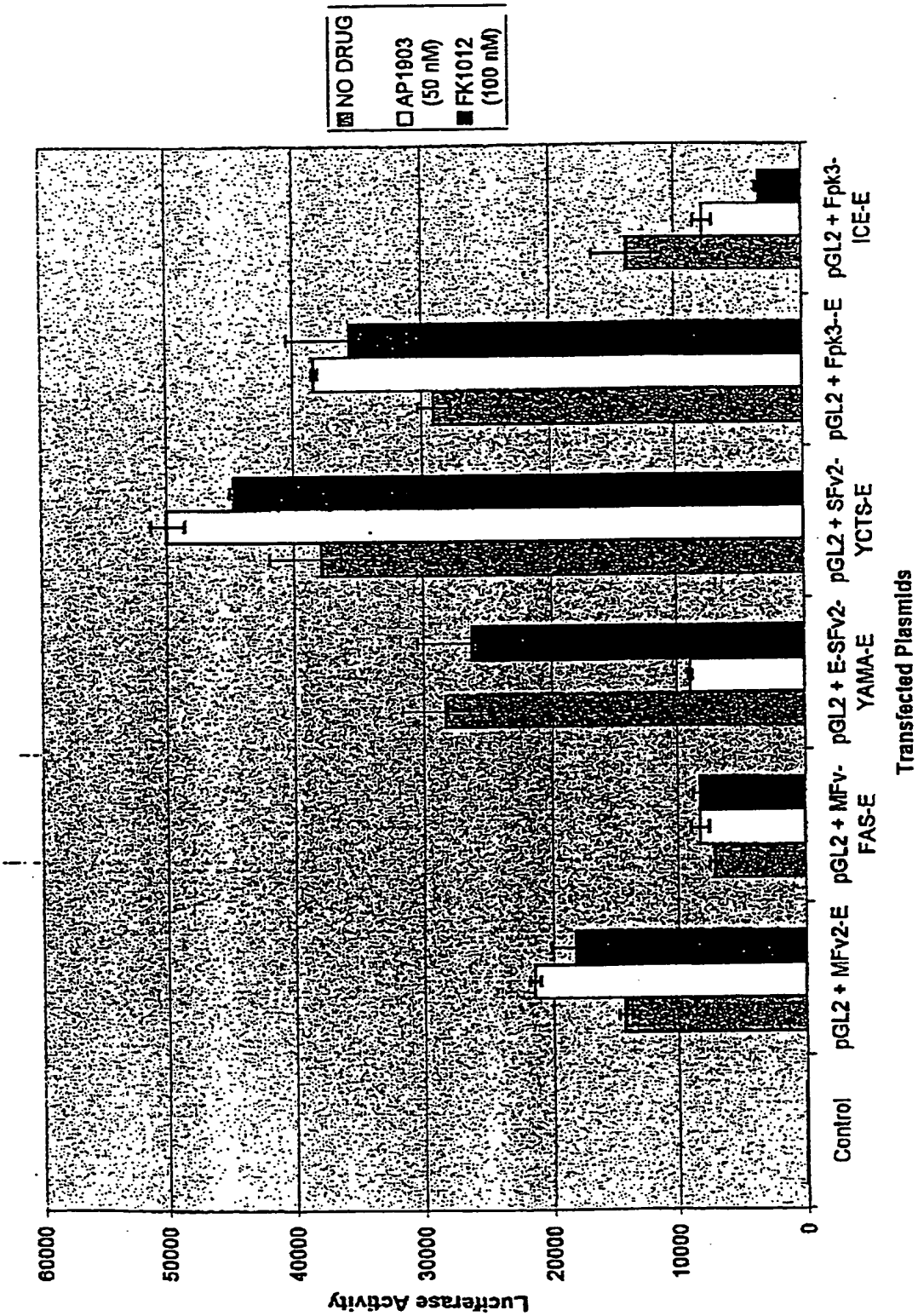
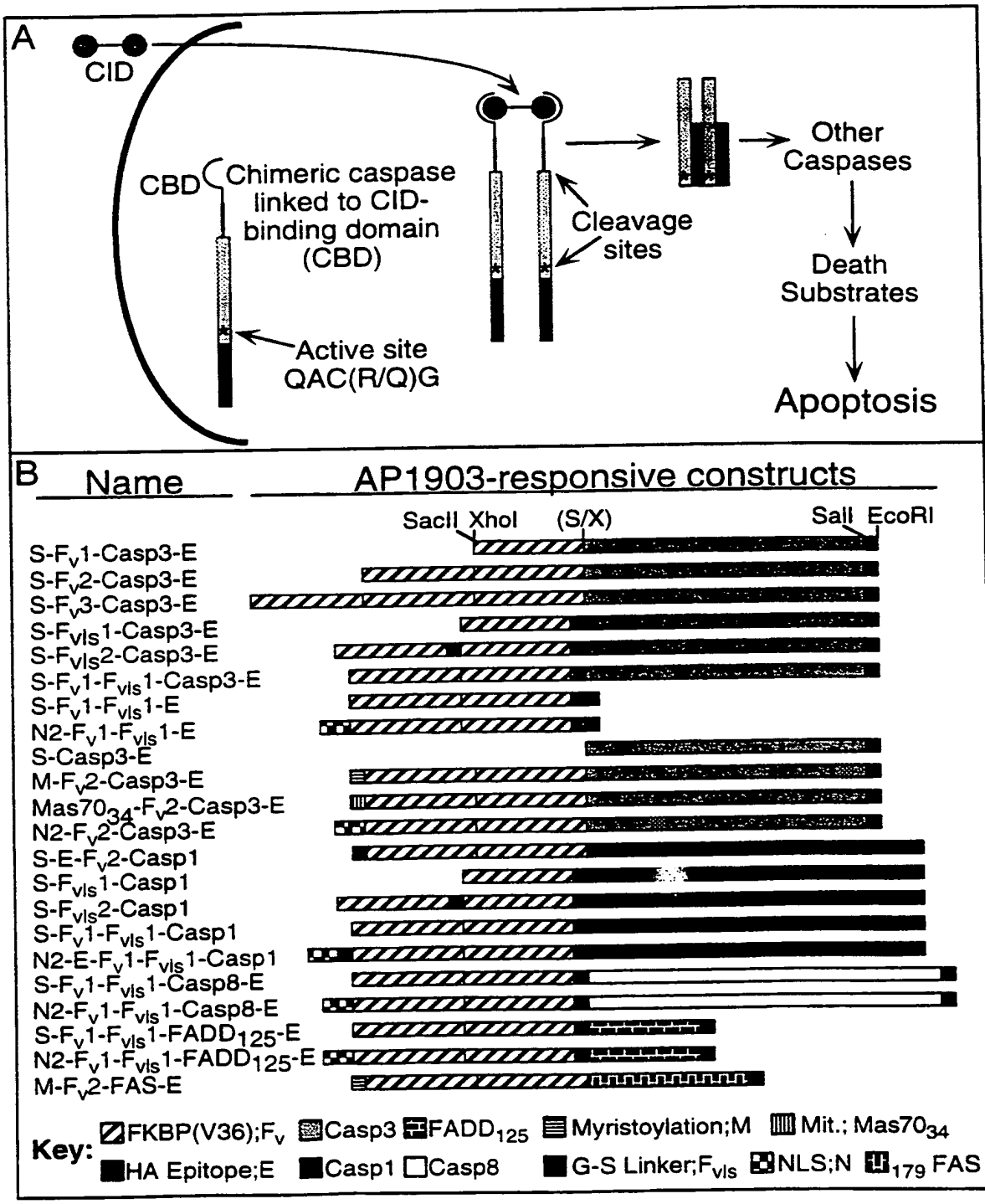
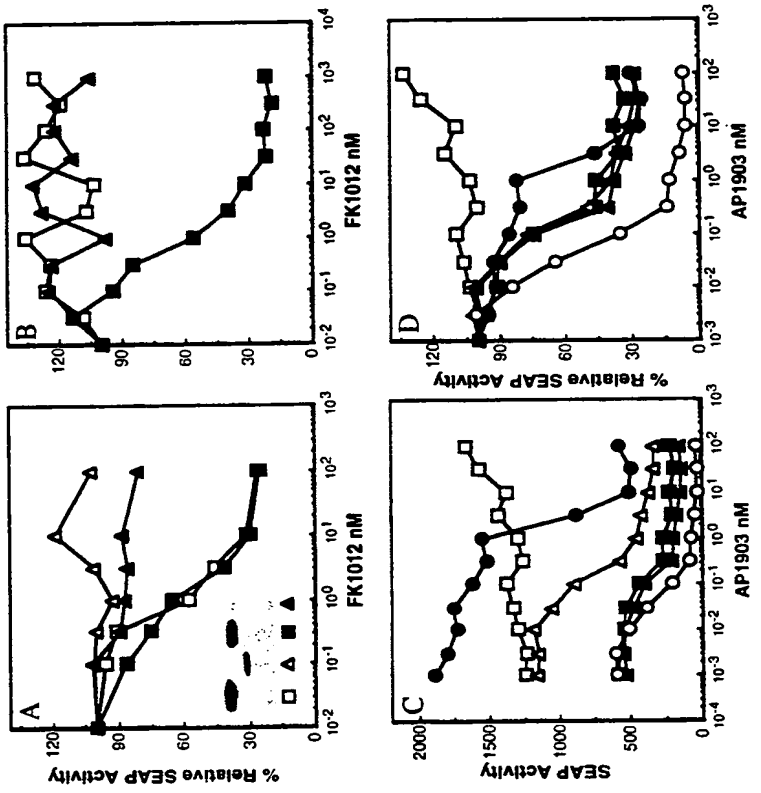

CID-Mediated Apoptosis in BPH derived JD SMC
NO DRUG
AP1903 (50 nM)
FK1012 (100 nM)
Luciferase Activity
60000
50000
40000
30000
20000
10000
0
Control
pGL2 + MFv2-E
pGL2 + MFv-FAS-E
pGL2 + E-SFv2-YAMA-E
pGL2 + SFv2-YCTS-E
pGL2 + Fpk3--E
pGL2 + Fpk3-ICE-E
Transfected Plasmids

FIG 23 (FIG 24 on Reverse)



Final Size: 50% or 11.1cm

Fig 25 (Fig 26 on Reverse)



Final Size: 67%
or 7 cm

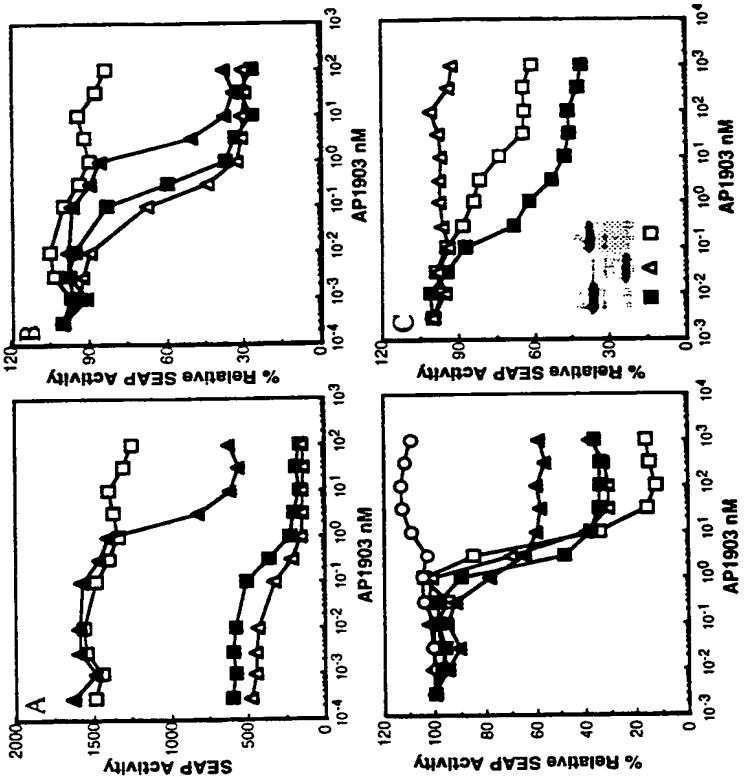


Fig 27

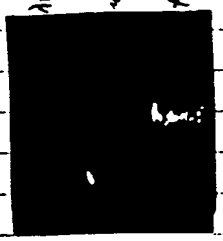
Final Size: 67%
or 6.9 cm

Generation of Ad-GFP-CMV-YM-E
Ad-GFP-CMV-E-~~ICE~~ US 99/06799

pAdTrack-CMV ~ Fv1s1 — YM-E
~E-Fv1-Fv1s1-ICE-st

① cut pAdTrack-CMV with EcoR V + Not I

1 ug/2 ul (pAdTrack-CMV) + 1 ul Buffer H
+ 1 ul EcoRV + 1 ul Not I + H_2O 5 ul
37°C, 2 hr.



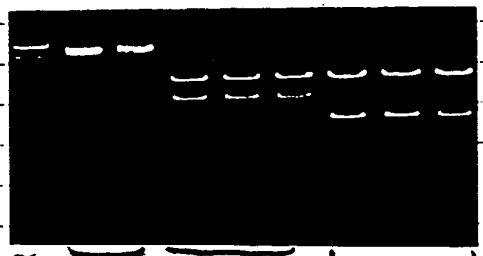
pAdTrack-CMV
9220 bp

② cut pSH1/S-Fv1s1-YM-E with Not I + EcoRI
(get 2.2kb Fv1s1-YM-E)
2 ug/1.2 ul (pSH1/S-Fv1s1-YM-E + 2 ul EcoRI
+ 2 ul Buffer H, + H_2O 14.8 ul, 37°C, 1 hr.

or (E-Fv1-Fv1s1-ICEst)
2 ug/3.2 ul
12.8 H_2O

Blunt
+ 10 uM dNTP 2 ul (10 uM/each) + 2 ul Buffer B
14 ul H_2O + 4U/2 ul Klenow, 25°C, 30 min; + EDTA → 10 mM
Inactivate Klenow by heating at 75°C, 10 min. phenol:chloroform extraction
~~for Gene Clean~~

Then + 2 ul Not I, 37°C, 1 hr.



③ Run gel, cut bands, pool together, Gene Clean. Elute in 40 ul

④ Ligation

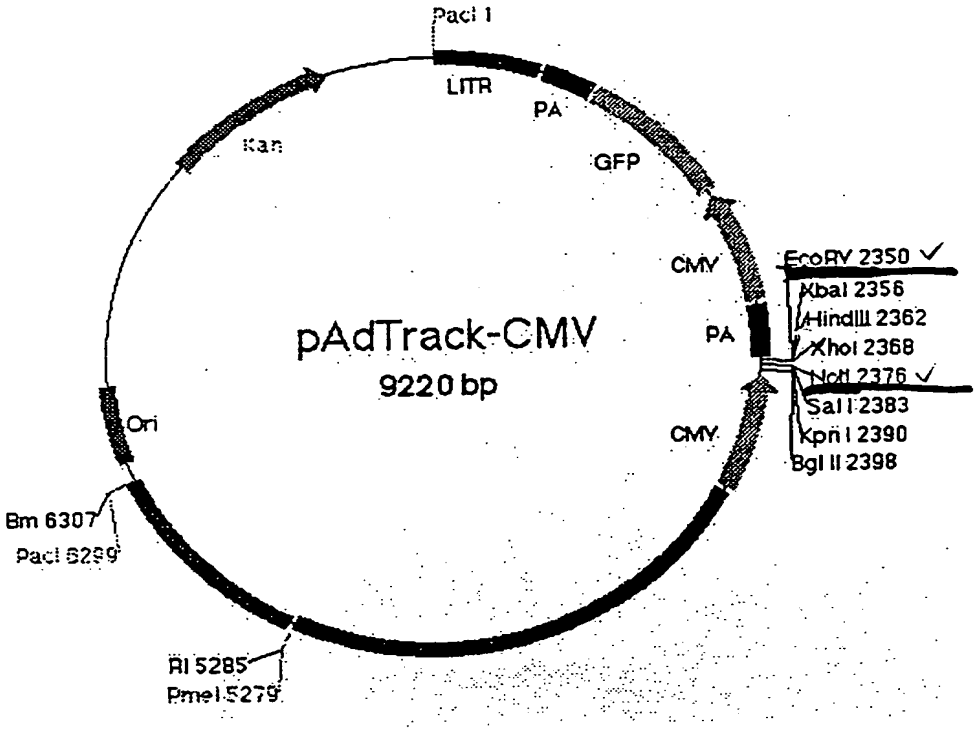
Eluted 16 ul + 2 ul 10X Ligation Buffer
+ 2 ul T4 ligase, 16°C, overnight.

pSH1/S-E-Fv1-Fv1s1-ICEst
+Not I
+Sal I

⑤ Transformation

⑥ Miniprep with Qiagen kit.

FIG 28


PacI 1
LITR
PA
GFP
CMV
PA
CMV
Kan
Ori
pAdTrack-CMV
9220 bp
EcoRV 2350
XbaI 2356
HindIII 2362
XhoI 2368
NotI 2376
SalI 2383
KpnI 2390
BglII 2398
Bm 6307
PacI 6299
RI 5285
PmeI 5279

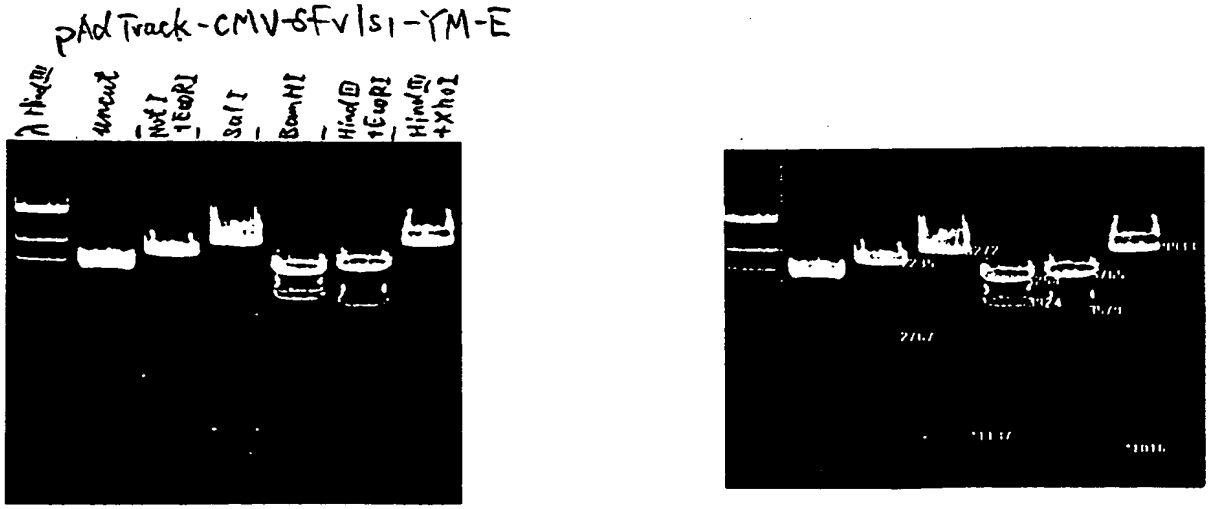
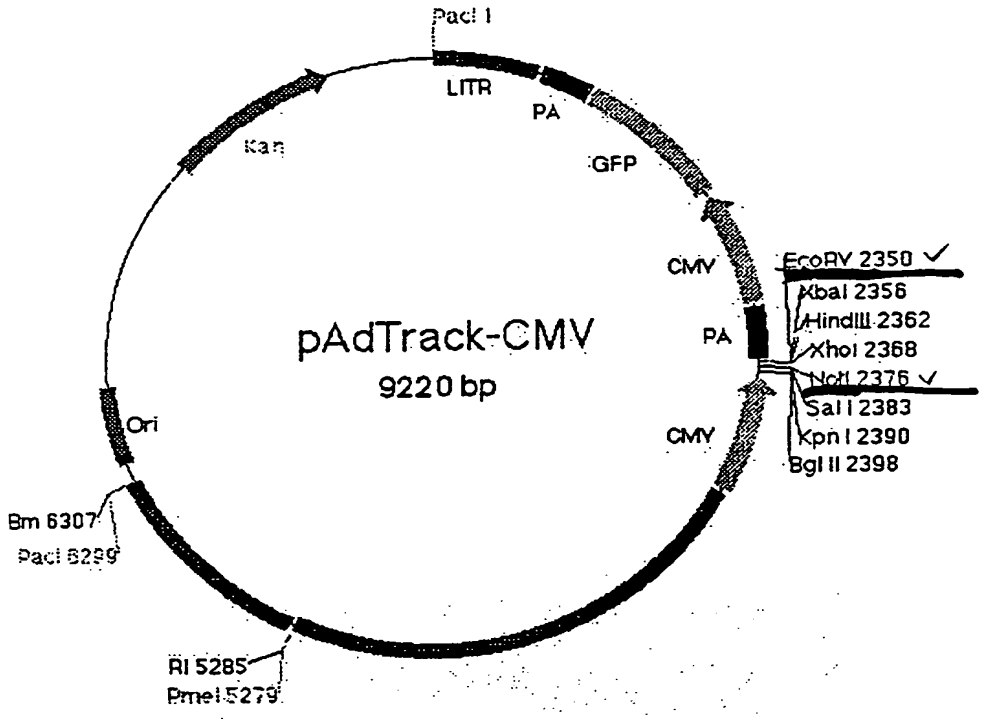

pAdTrack-CMV-SFvIs1-YM-E
λ HindIII
uncut
-NotI +EcoRI
-SalI -
-BamHI -
-HindIII +EcoRI
-HindIII +XhoI

FIG-29

JPEG image 511x431 pixels



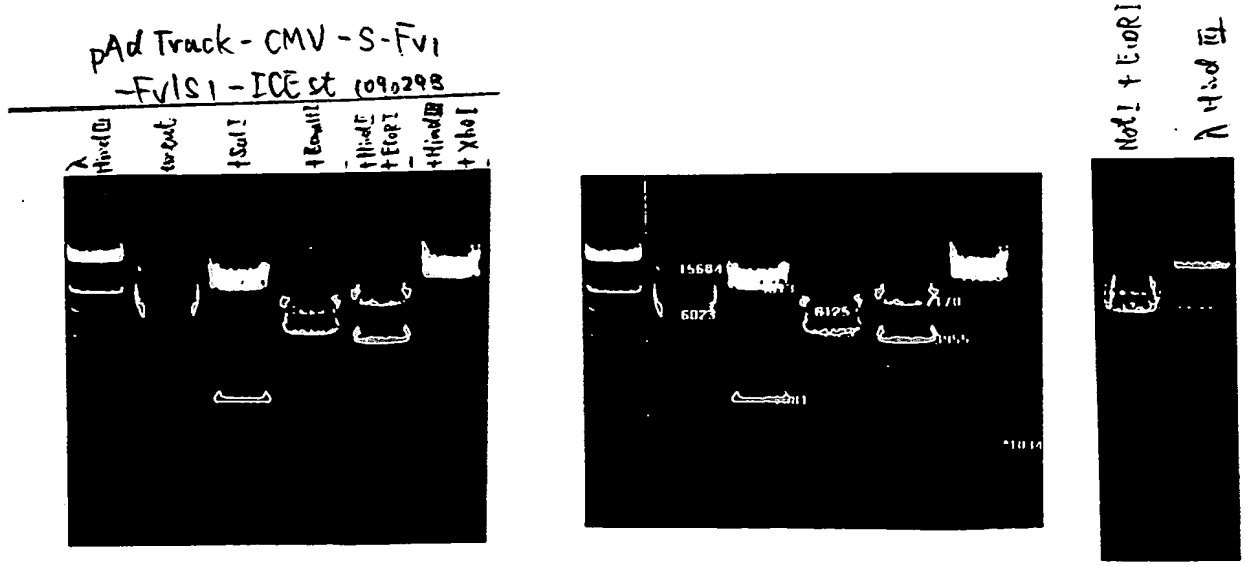
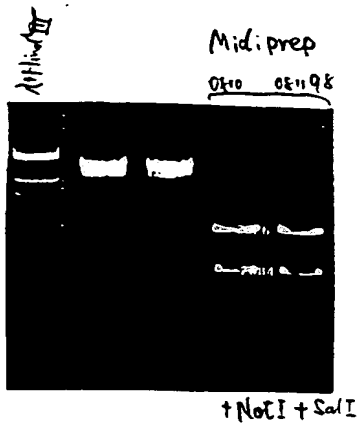


FIG 30

A3


Midiprep
†NotI †SalI

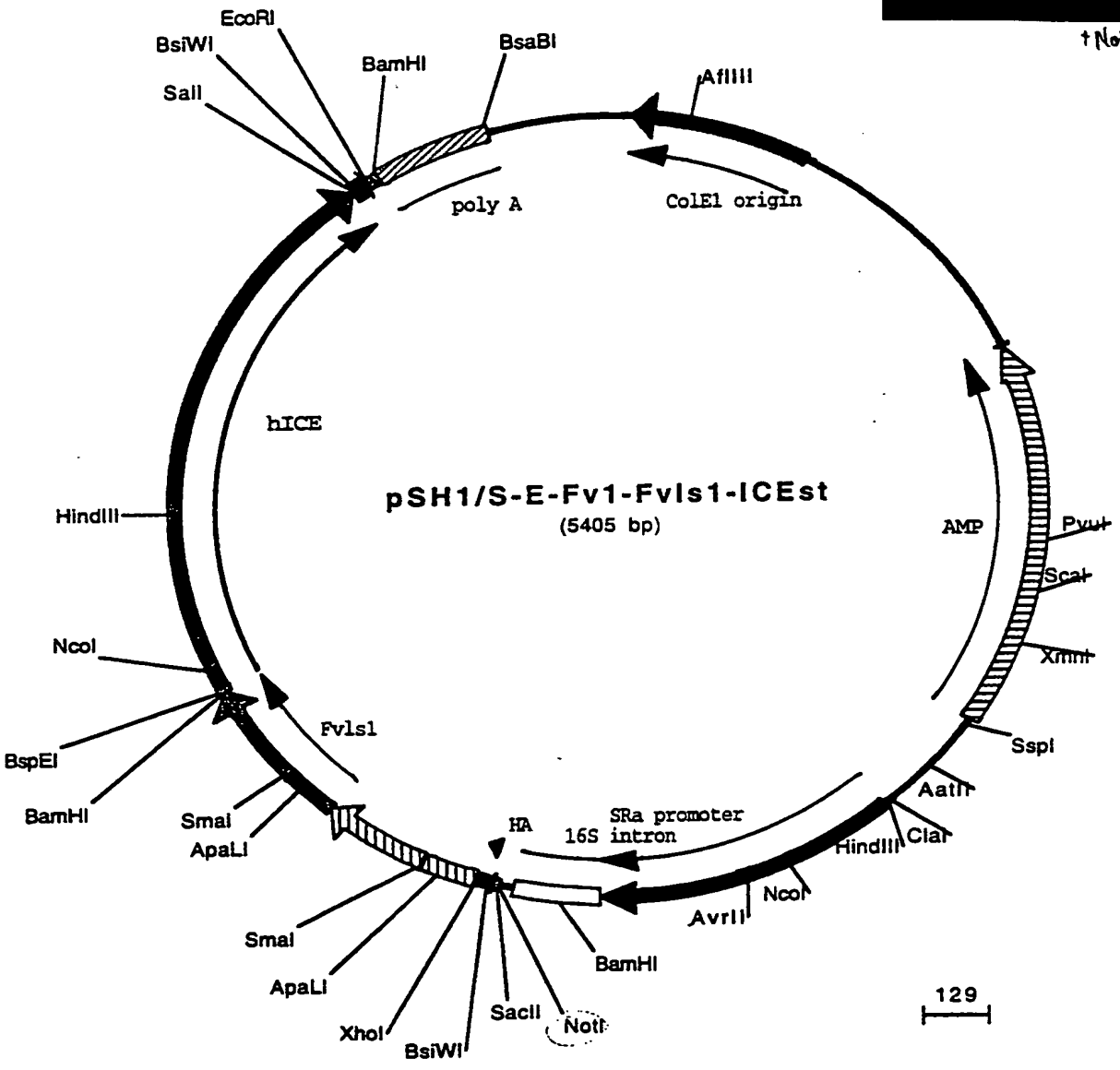

pSH1/S-E-Fv1-Fvls1-ICEst
(5405 bp)
EcoRI
BsiWI
SalI
BamHI
BsaBI
AflIII
poly A
ColE1 origin
hICE
HindIII
NcoI
BspEI
BamHI
SmaI
ApaLI
Fvls1
HA
SRa promoter
16S intron
SmaI
ApaLI
XhoI
BsiWI
SacII
NotI
BamHI
AvrII
NcoI
HindIII
ClaI
AatII
SspI
XmnI
ScaI
PvuI
AMP
129

FIG 31

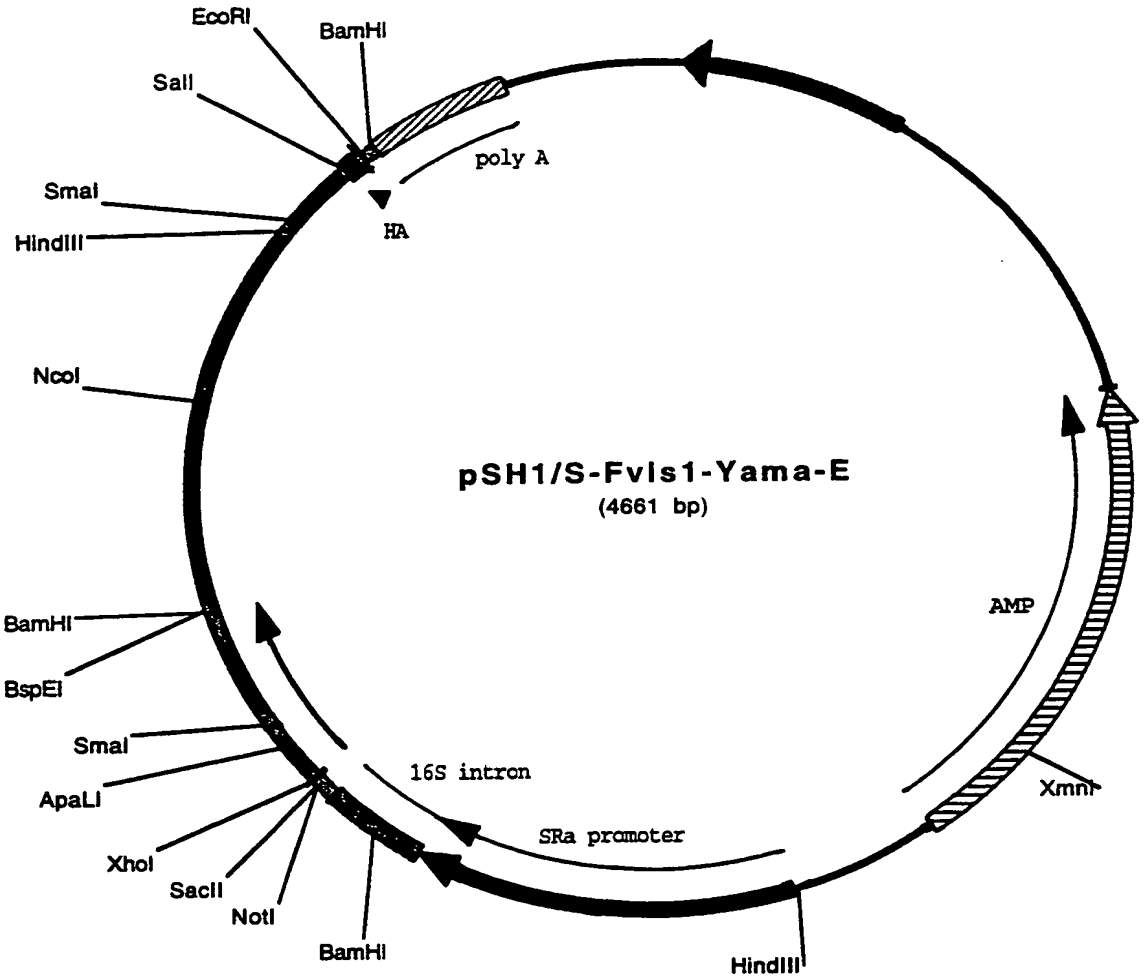

EcoRI
BamHI
SalI
poly A
SmaI
HA
HindIII
NcoI
pSH1/S-Fvls1-Yama-E
(4661 bp)
111
AMP
BamHI
BspEI
SmaI
16S intron
ApaLI
Xmnl
SRa promoter
XhoI
SacII
NotI
BamHI
HindIII

FIG 32

Generation of Ad-GFP-CMV-YM-E
Ad-GFP-CMV-E-ICE

① Co-transform E. coli BJ5183 with pAdEasy-1 and pAdTrack-CMV-Fv1s1-YM-E or pAdTrack-CMV-E-Fv1-Fv1s1-ICE st

a. Linearize the shuttle plasmids with Pme I
1ug of pAdTrack-CMV-Fv1s1-YME
or pAdTrack-CMV-E-Fv1-Fv1s1-ICEst
+ 1ul Buffer + H_2O → 9 ul, + 1ul Pme I

37°C, 2hr.

phenol-chloroform extraction, ethanol precipitation and resuspend in 6 ul H_2O.

B. Co-transformation: with 100 ng pAdEasy-1
20 ul of BJ5183
2.0 mm cuvettes
at 2500V, 200 Ohms, 25 uFD

C. Selection:
pick 10 smallest colonies, miniprep, check with Pac I.
re-transform-XL1-Blue with the correct plasmid, miniprep, recheck with Pac I.
Midiprep.

d. Transfect 293 cells with Fugiene.

90% confluence 293 cells in 6-well-plate,
4 ug plasmid/6 ul Fugiene/well.

FIG 33

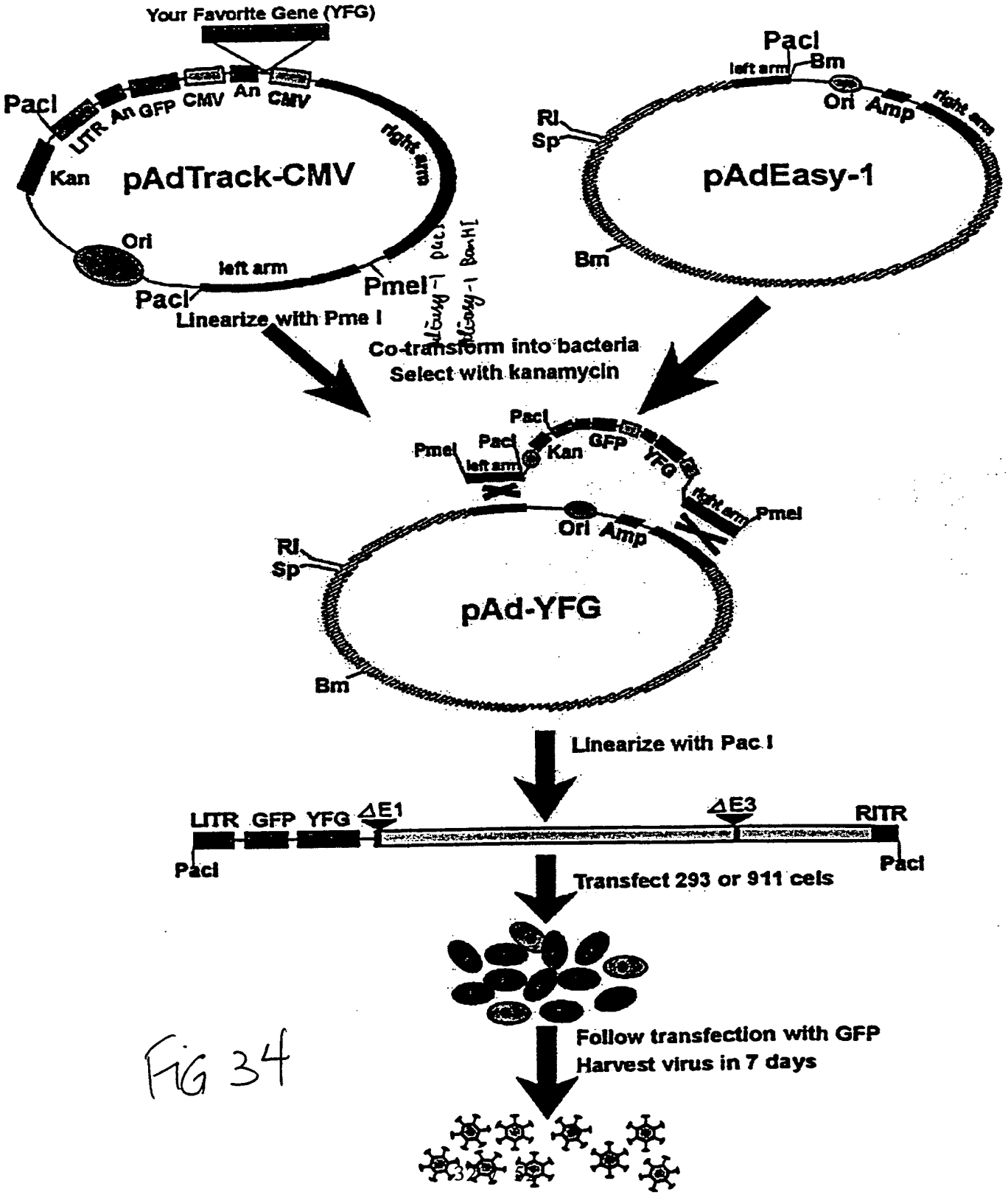

Your Favorite Gene (YFG)
PacI
LITR An GFP CMV An CMV
Kan
pAdTrack-CMV
right arm
Ori
left arm
PacI
PmeI
Linearize with Pme I
PacI
left arm
Bm
Ori Amp
right arm
RI
Sp
pAdEasy-1
Bm
Co-transform into bacteria
Select with kanamycin
PacI
PmeI
PacI
left arm
Kan
GFP
YFG
right arm
PmeI
Ori Amp
RI
Sp
pAd-YFG
Bm
Linearize with Pac I
LITR GFP YFG ΔE1
ΔE3
RITR
PacI
PacI
Transfect 293 or 911 cels
Follow transfection with GFP
Harvest virus in 7 days

FIG 34

pAdEasy-1
33414 bp
Pacl 1
BamHI 8
Ori
Amp
Ad5dE1I3
EcoRI 27465
SpeI 27216
BamHI 21696

FIG 35

pAd Easy-1-Track-CMV-ICE-E
-YAMA-E

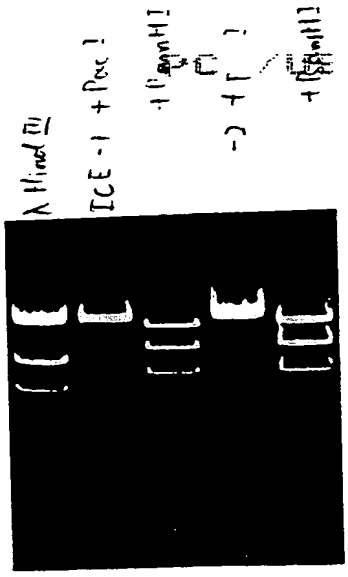
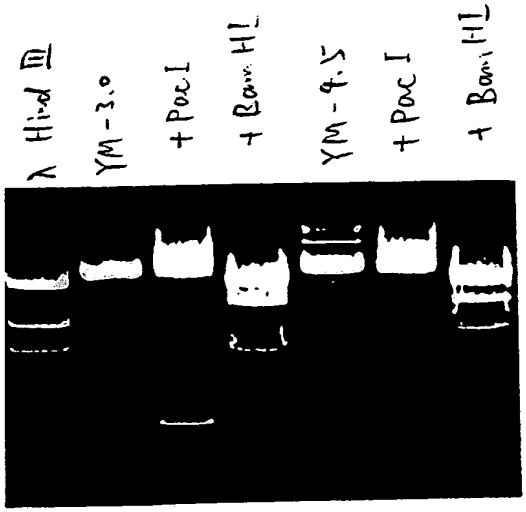


FIG 36



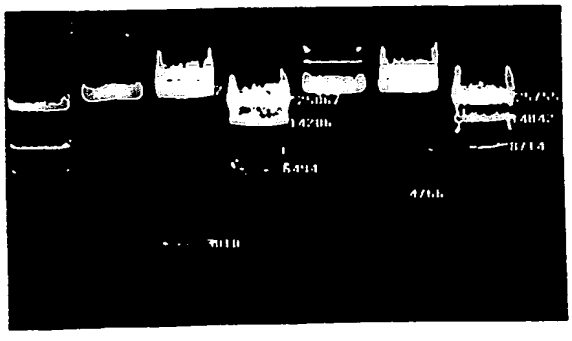
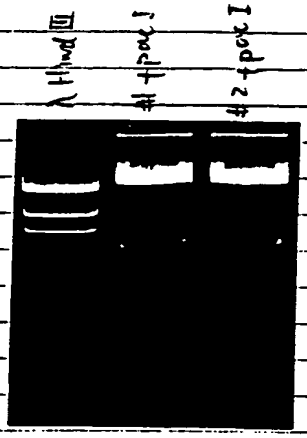


FIG 37 Generation of Ad-CMV-E-~~FEG~~ US 99/06799

Construction of pShuttle-CMV-E-Fv1-Fv1s1-ICEst and Ad-CMV-E-ICE

① cut pShuttle-CMV with EcoRV + Not I

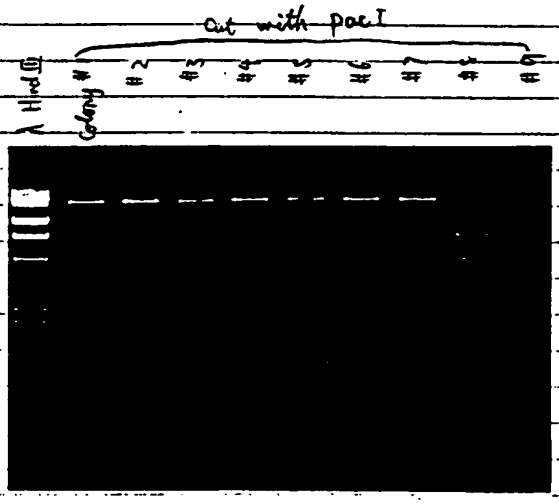
1ug/2 ml (pShuttle-CMV) + 1 ml Buffer H
+ 1 ml EcoRV + 1 ml Not I + H_2O 5 ml
37°C, 2 hr.



② cut pSH/S-E-Fv1-Fv1s1-ICEst with
Not I + EcoRI (cut E-Fv1-Fv1s1-ICEst)

3 ug/4 ml + 3 ml Buffer H + 3 ml EcoRI
+ H_2O 21 ml, 37°C, 1 hr.

Blunt
+ 10 mM dNTP 15 ml (10 mM/each) + 3 ml Buffer B
+ 3 ml H_2O + 7 ml T4 DNA Polymerase, 25°C, 30';
+ EDTA → 10 mM; 75°C, 10'
phenol-chloroform extraction (twice)
ethanol precipitation → 24 ml H_2O



Then
+ 3 ml Buffer H + 3 ml Not I
37°C, 2 hr.

③ Run Gel, cut bands, pool together,
GeneClean, Elute in 40 ml H_2O

④ Ligation

Eluted 16 ml + 2 ml 10X Ligation Buffer
+ 2 ml T4 Ligase 25°C overnight

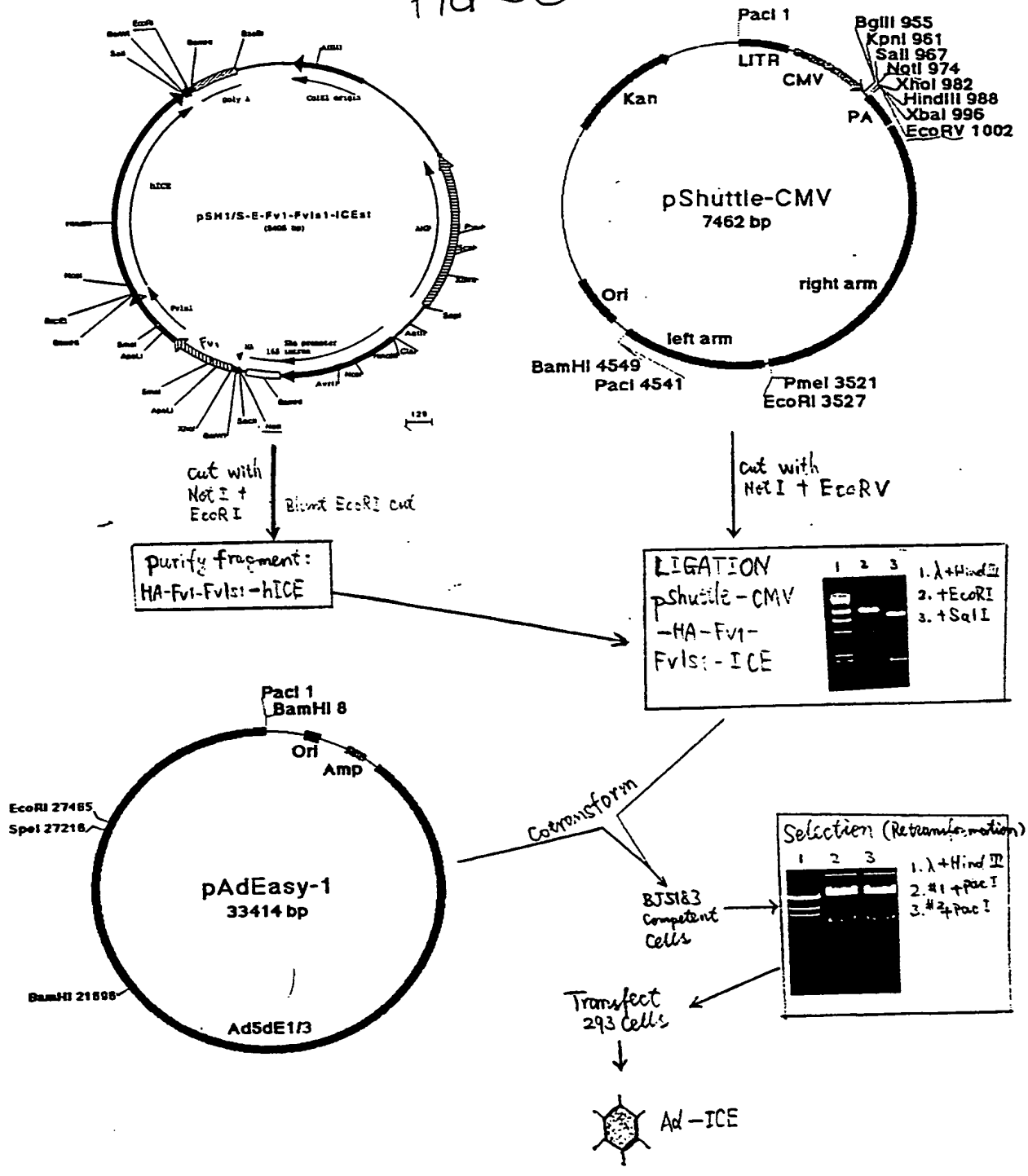
⑤ Transformation

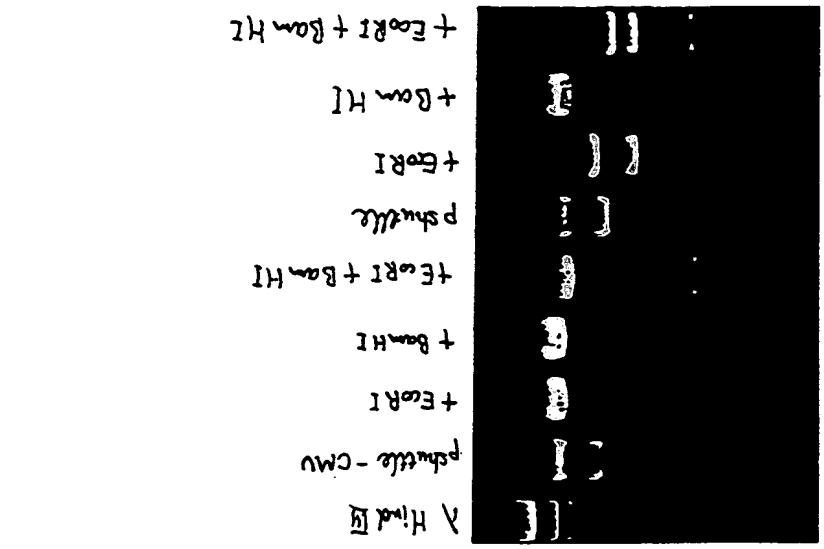
⑥ Miniprep + Restriction Enzyme checking

⑦ cut pShuttle-CMV-E-Fv1-Fv1s1-ICEst with PmeI (1/5 of a miniprep), Coretransform E. coli BJ5183 with 100 ng pAdEasy-1. Miniprep + pacI checking (photo above)

⑧ Chose #2, retransform XL-1 Blue, Miniprep, +pacI; Midiprep, + pacI checking (small photo abo

FIG 38


pSH1/S-E-Fv1-Fvls1-ICEs1
PacI 1
BglII 955
KpnI 961
SalI 967
NotI 974
XhoI 982
HindIII 988
XbaI 996
EcoRV 1002
LITR
CMV
PA
Kan
pShuttle-CMV
7462 bp
Ori
right arm
left arm
BamHI 4549
PacI 4541
PmeI 3521
EcoRI 3527
Cut with NotI + EcoRI
Blunt EcoRI cut
Cut with NotI + EcoRV
Purify fragment: HA-Fv1-Fvls1-hICE
LIGATION pShuttle-CMV -HA-Fv1- Fvls1-ICE
1. λ+HindIII
2. +EcoRI
3. +SalI
PacI 1
BamHI 8
Ori
Amp
EcoRI 27485
SpeI 27216
pAdEasy-1
33414 bp
BamHI 21595
Ad5dE1/3
Cotransform
BJ5183 Competent Cells
Selection (Retransformation)
1. λ+HindIII
2. #1 + PacI
3. #2 + PacI
Transfect 293 Cells
Ad-ICE



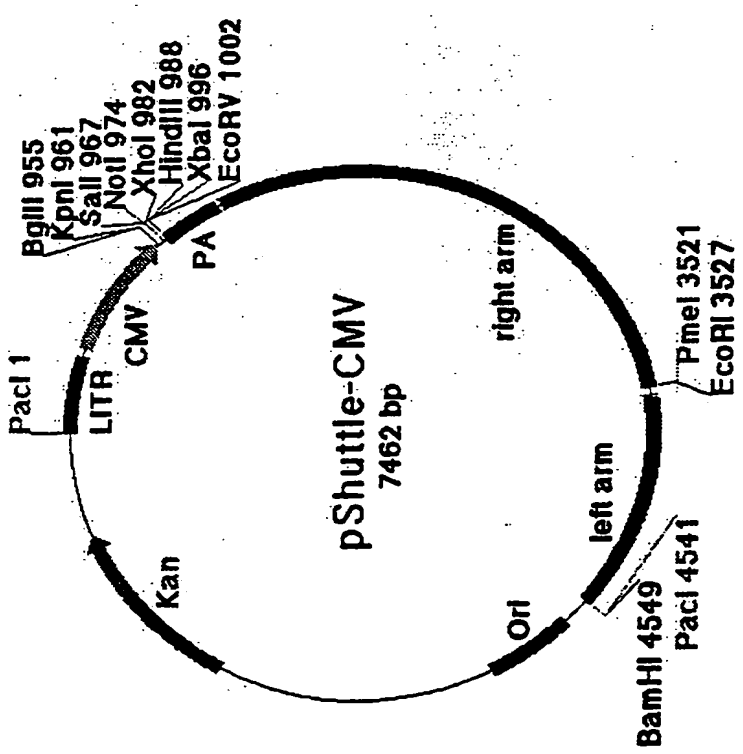


Fig 39

FIG 40

Luciferase Assay of pSuitible-CMV -FV1-FMS1-ICE-E 0104-010799

① 1X10^5 JD-20 cells / well (6 well - Nalge)
in 10% RPMI media with 5% FBS
Incubate 24 hrs

② Transfection: 2ug pGL2 2ug ICE per well
Fugene 2ul / ug DNA

Incubate 6 hrs, add AP1903 at 10 nM.

Incubate 24 hrs.

③ lysis — luminometer

8 JAN 99 AM 10:59 V. 1.1

MEAS. TIME(s) : 15.0

SAMPLE		RLU	% C.V.	
	1 1	294		Control
	1 2	291		
MEAN		292	0.8	
	2 1	2681959		
	2 2	2769999		
MEAN		2725979	2.3	Control + pGL2
	3 1	208762		
	3 2	223711		
MEAN		216237	4.9	pGL2 + ICE
	4 1	22084		
	4 2	22859		
MEAN		22471	2.4	pGL2 + ICE + AP1903

FIG 41

JD-29, T-C2, T-C2G, LNCaP — Adeno-TM or Adeno-ICE

① Plating cells: 24 well-plate

JD-29 — 20000/well
T-C2 > 10000/well > in 1ml RPMI 1640 Media (5% FBS)
T-C2G >
LNCaP — 30000/well > in 1ml DMEM Media (+5% FBS, 5% Nuserm, Insulin DEET

incubate JD 24 hrs — Cell number doubling — 40000
T-C2, TC2G — 48 hrs. — 40000
LNCaP — 72 hrs — 60000

② Infection:

For JD: ICE 1×10^7 iu/ml	MOI	1	2	4	8	16	
	Add	4	8	16	32	64	ul
YM 2×10^7 iu/ml	MOI	2.5	5	10	20	40	
		5	10	20	40	80	ul
		1:10					
For T-C2 ICE 5×10^7 iu/ml	MOI	2.5	5	10	20	40	
+T-C2G	Add	2	4	8	16	32	
YM 5×10^7 iu/ml	MOI	2.5	5	10	20	40	
	Add	2	4	8	16	32	
For LNCaP		3	6	12	24	48	

After 4 hrs, Add AP 1903 → 50 nM, incubate 24 hrs.

③ Fix with 1% Glutaraldehyde — PBS, 15';
Stain 0.5% Crystal violate, 20';
Wash with H2O, 30';
Resolve with Sorenson's Solution, 200 ul/well, 5';
Transfer 100 ul to each well of 96-well plate. Read OD at 570 nM.

T-C2G Cells Treated with Ad-YAMA or Ad-ICE + AP1903 or AP20187

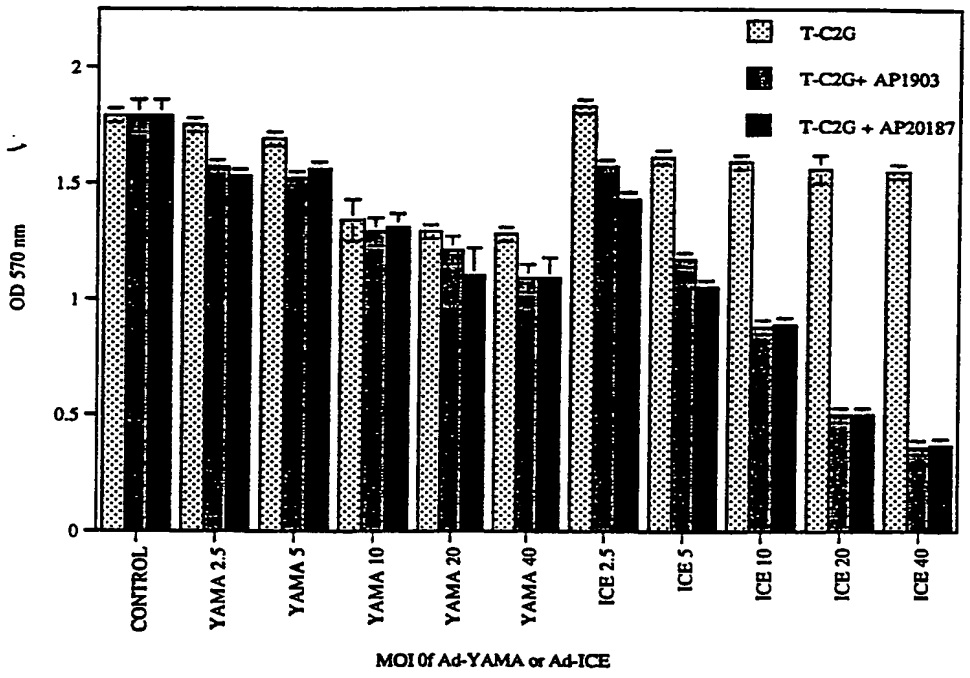


FIG 42

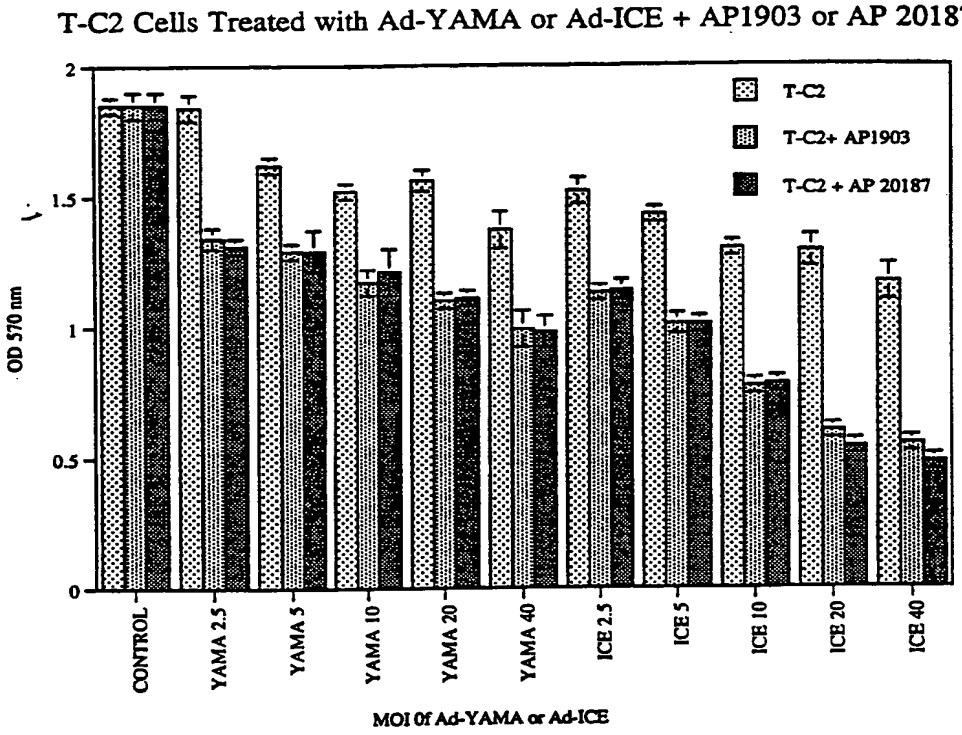

T-C2 Cells Treated with Ad-YAMA or Ad-ICE + AP1903 or AP 20187
T-C2
T-C2+ AP1903
T-C2 + AP 20187
OD 570 nm
2
1.5
1
0.5
0
CONTROL
YAMA 2.5
YAMA 5
YAMA 10
YAMA 20
YAMA 40
ICE 2.5
ICE 5
ICE 10
ICE 20
ICE 40
MOI Of Ad-YAMA or Ad-ICE

FIG 43

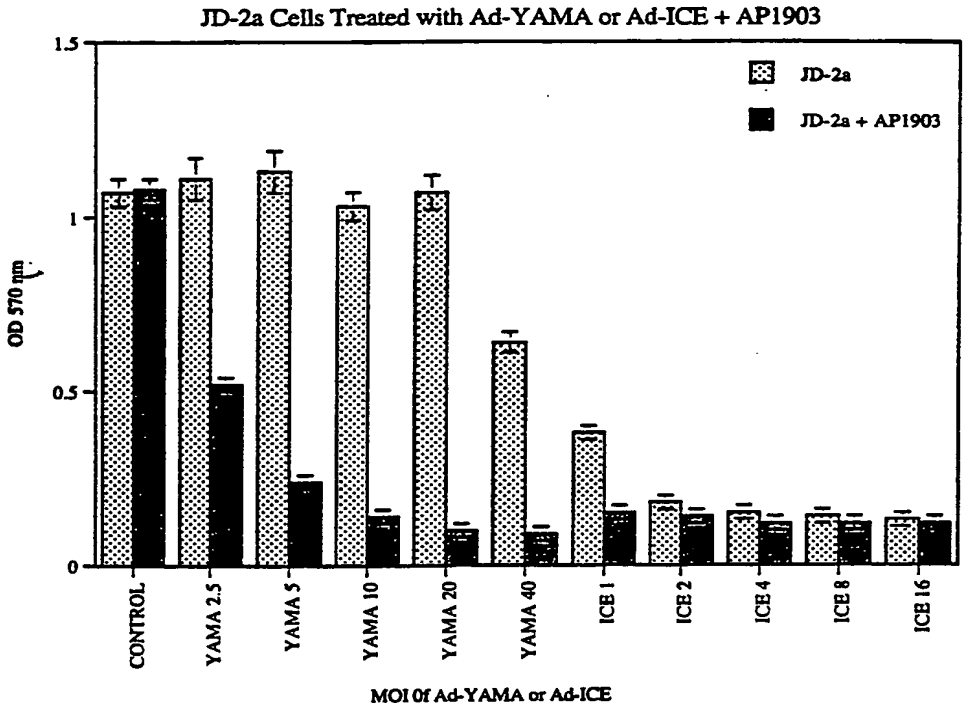

JD-2a Cells Treated with Ad-YAMA or Ad-ICE + AP1903
JD-2a
JD-2a + AP1903
OD 570 nm
1.5
1
0.5
0
CONTROL
YAMA 2.5
YAMA 5
YAMA 10
YAMA 20
YAMA 40
ICE 1
ICE 2
ICE 4
ICE 8
ICE 16
MOI 0f Ad-YAMA or Ad-ICE

FIG 44

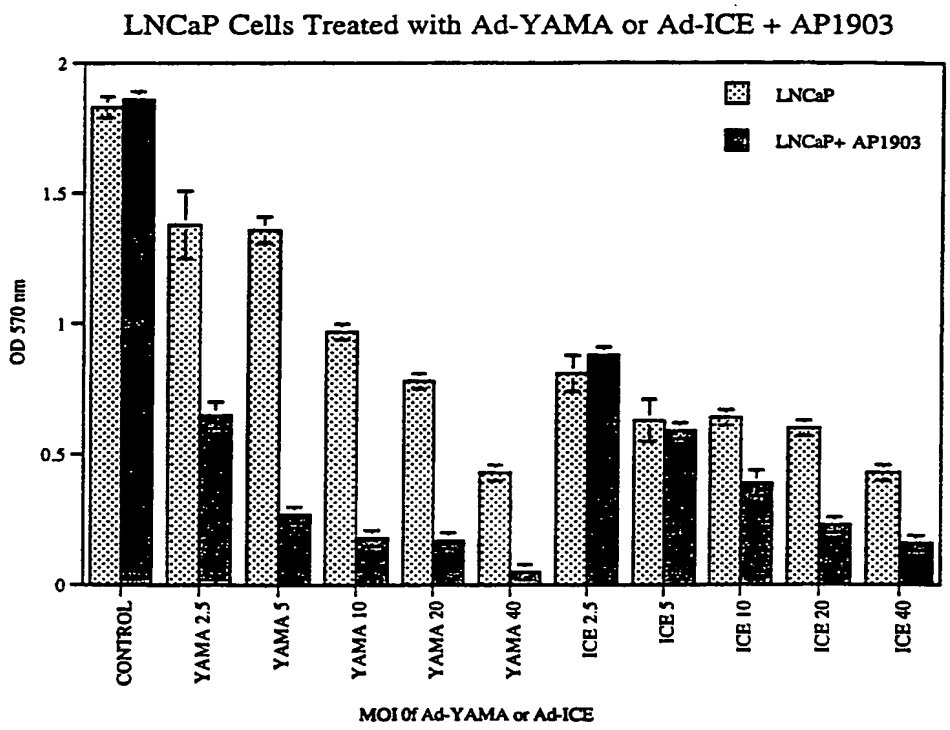

LNCaP Cells Treated with Ad-YAMA or Ad-ICE + AP1903
LNCaP
LNCaP+ AP1903
OD 570 nm
2
1.5
1
0.5
0
CONTROL
YAMA 2.5
YAMA 5
YAMA 10
YAMA 20
YAMA 40
ICE 2.5
ICE 5
ICE 10
ICE 20
ICE 40
MOI 0f Ad-YAMA or Ad-ICE

FIG 45

Western Blot 1103 – 1104

293 cells infected with Ad-YM or Ad-ICE

9% Resolving Gel ; Transfer – 400 mA , 2 hrs .

1:1000 α-HA11 – 1st Ab.

1 Ad-YM3
2 " + 1903 (100 nM)
3 " + 2-D-DCB (100 nM)
4 " + Both
5 Ad-ICE
6 " + 1903
7 " + 2-D-DCB
8 " + Both
9 Ad-YM4
10 " + 1903
11 " + 2-D-DCB
12 " + Both

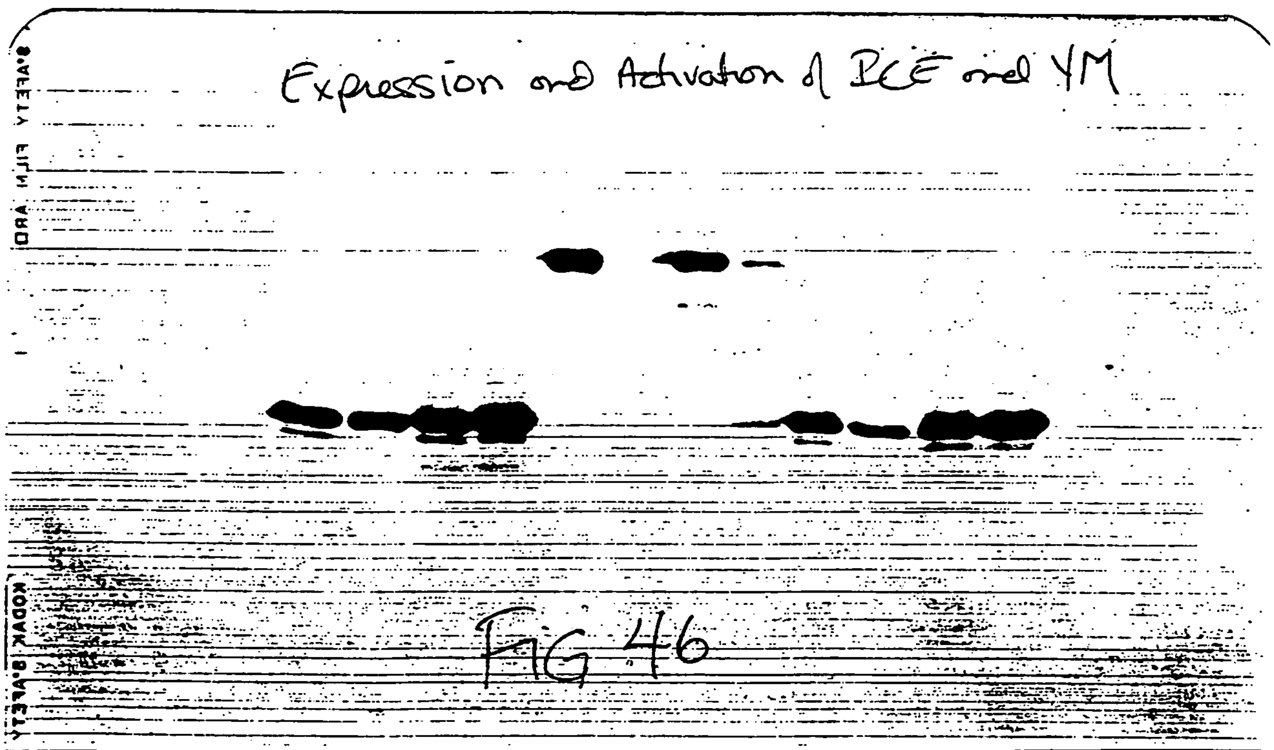


FIG 46

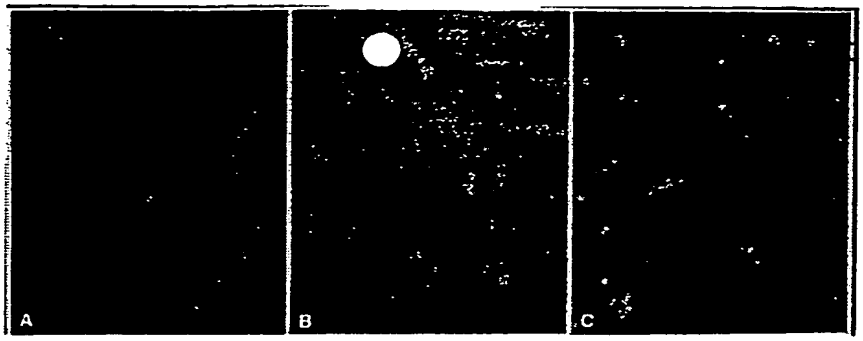


Fig 47

ADV-FKBP/ICE effectively kills PC-3 prostate cancer cells

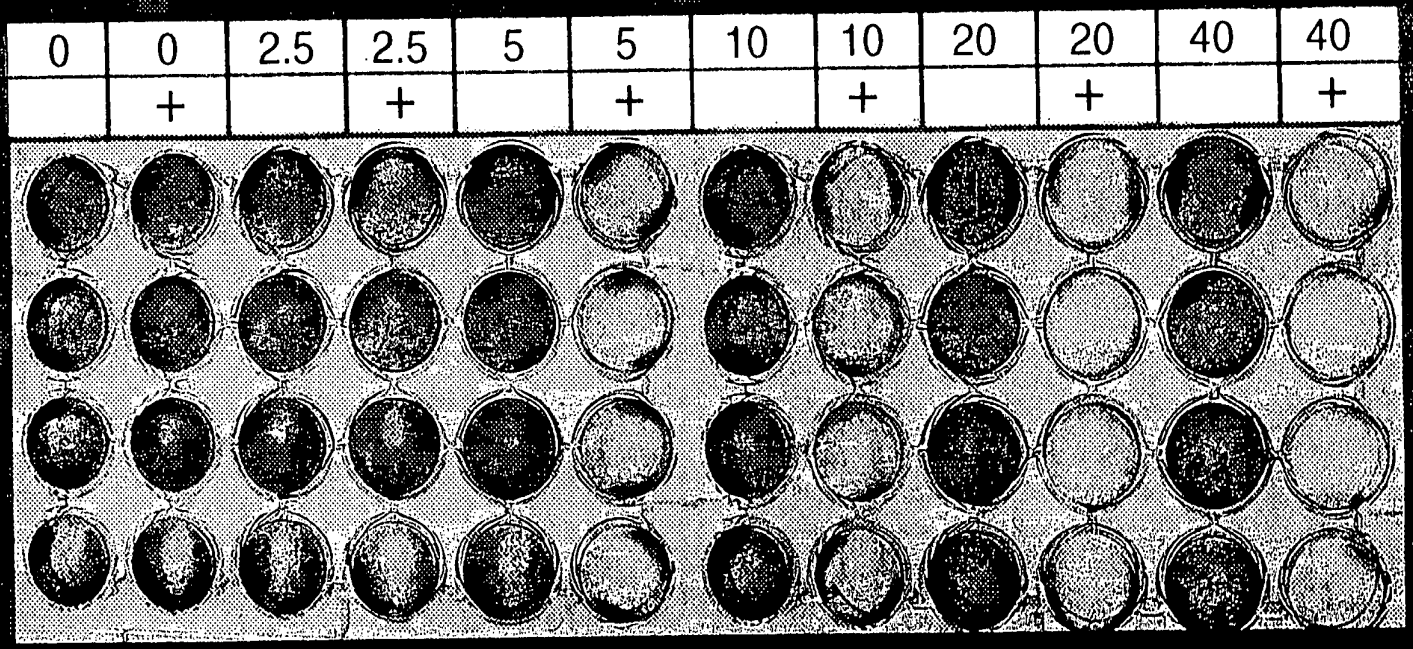


FIG 48

ADV-FKBP/ICE effectively kills JD-2a BPH cells

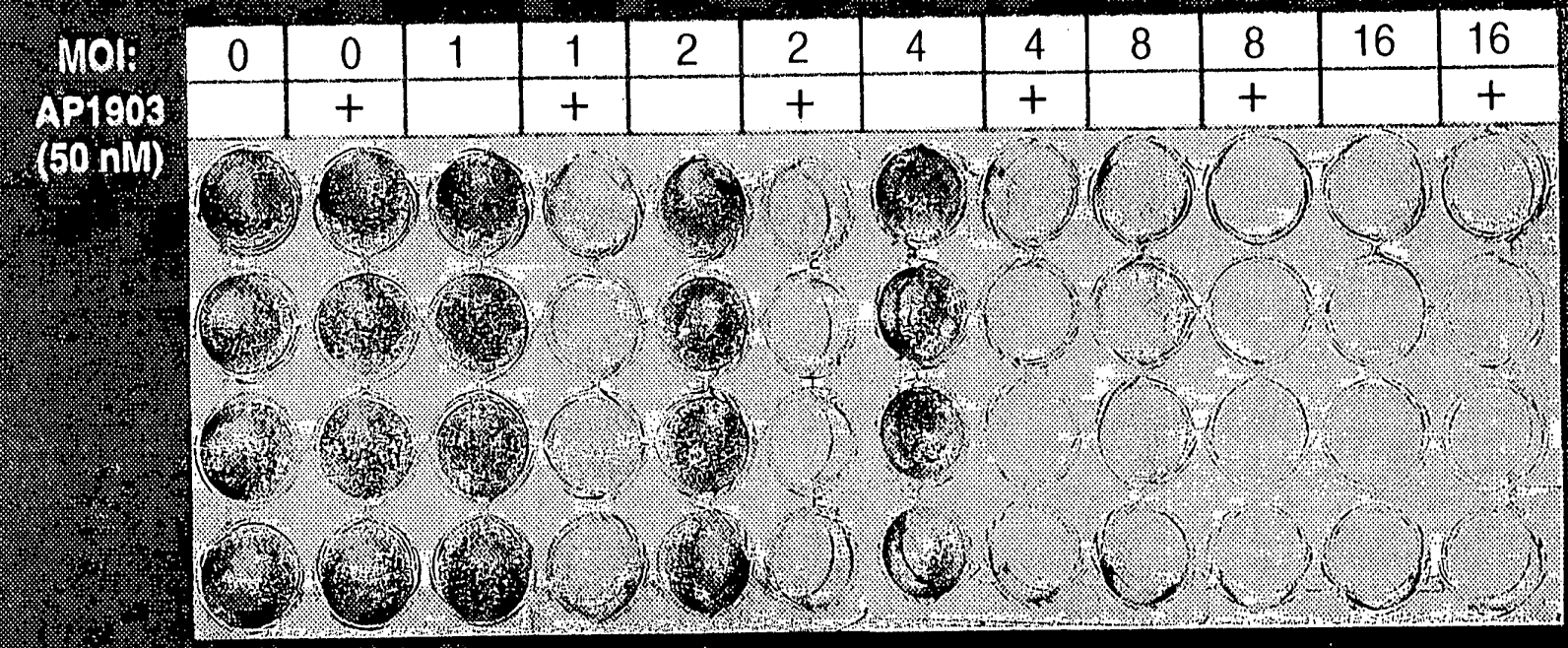
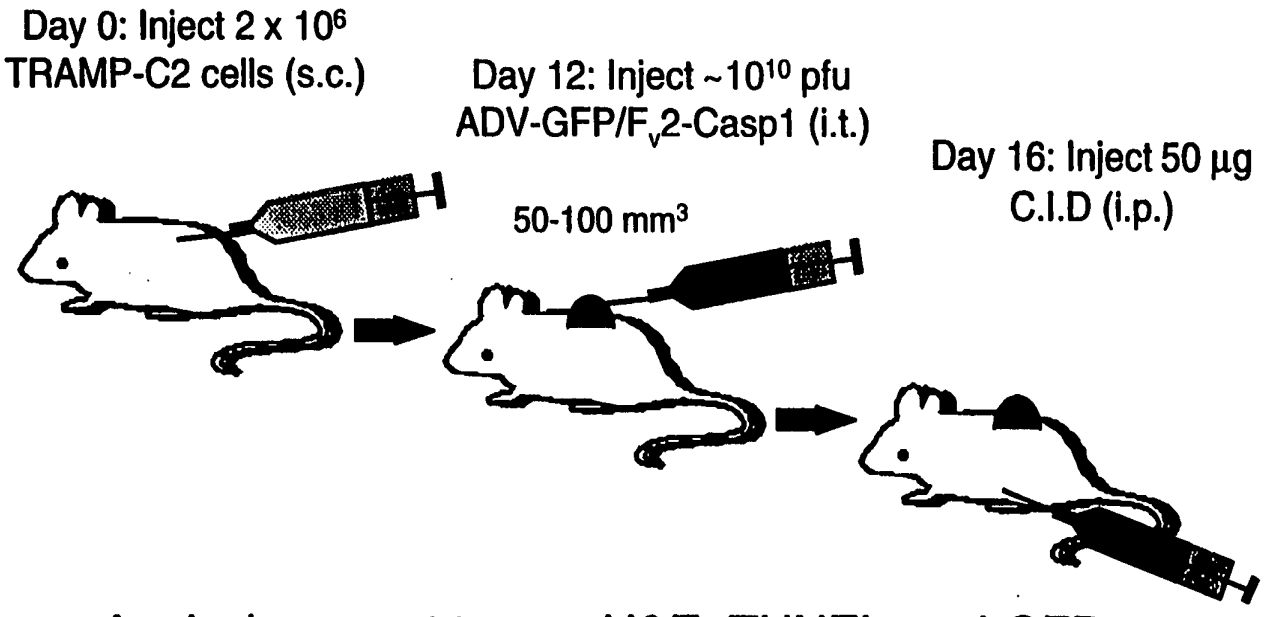


Fig 49

WO 99/50425
PCT/US99/06799
09/647418

Treatment of s.c. prostate adenocarcinoma in situ with CID-inducible caspases



Analysis: resect tumor, H&E, TUNEL, anti-GFP

FIG 50

ADV-FKBP/ICE effectively kills TRAMP-C2 cells in vivo

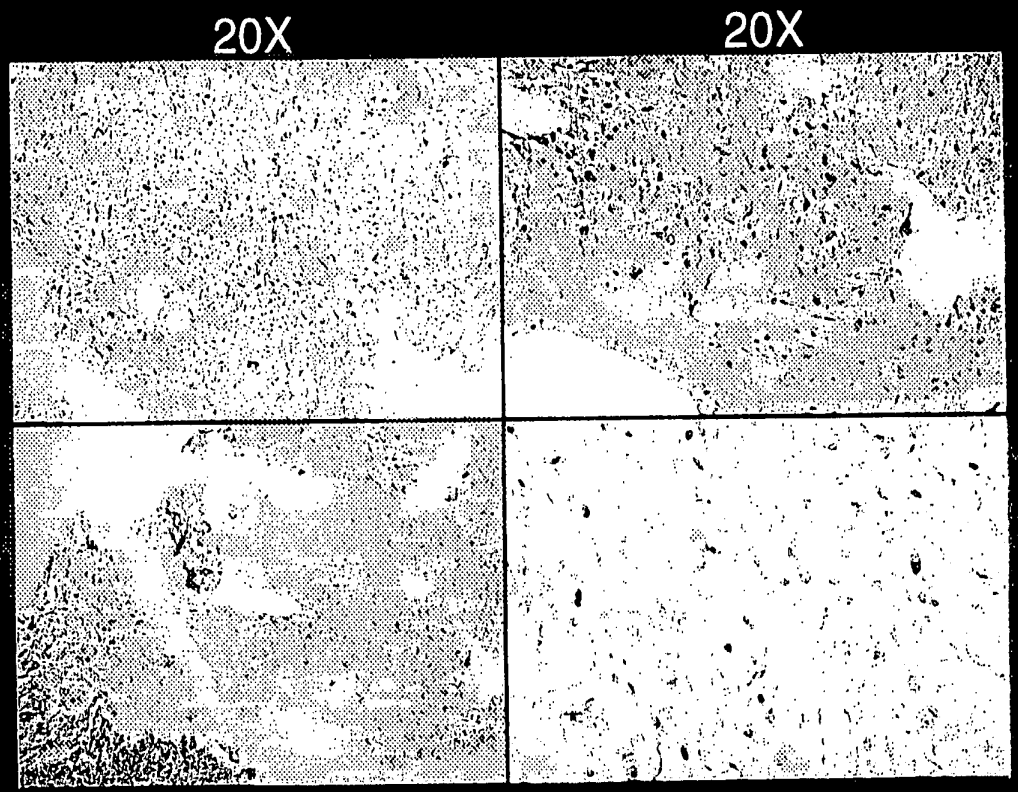


Fig 5I

WO 99/50425
PCT/US99/06799

ADV-FKBP/ICE + CID very effectively kills TRAMP-C2 cells in vivo

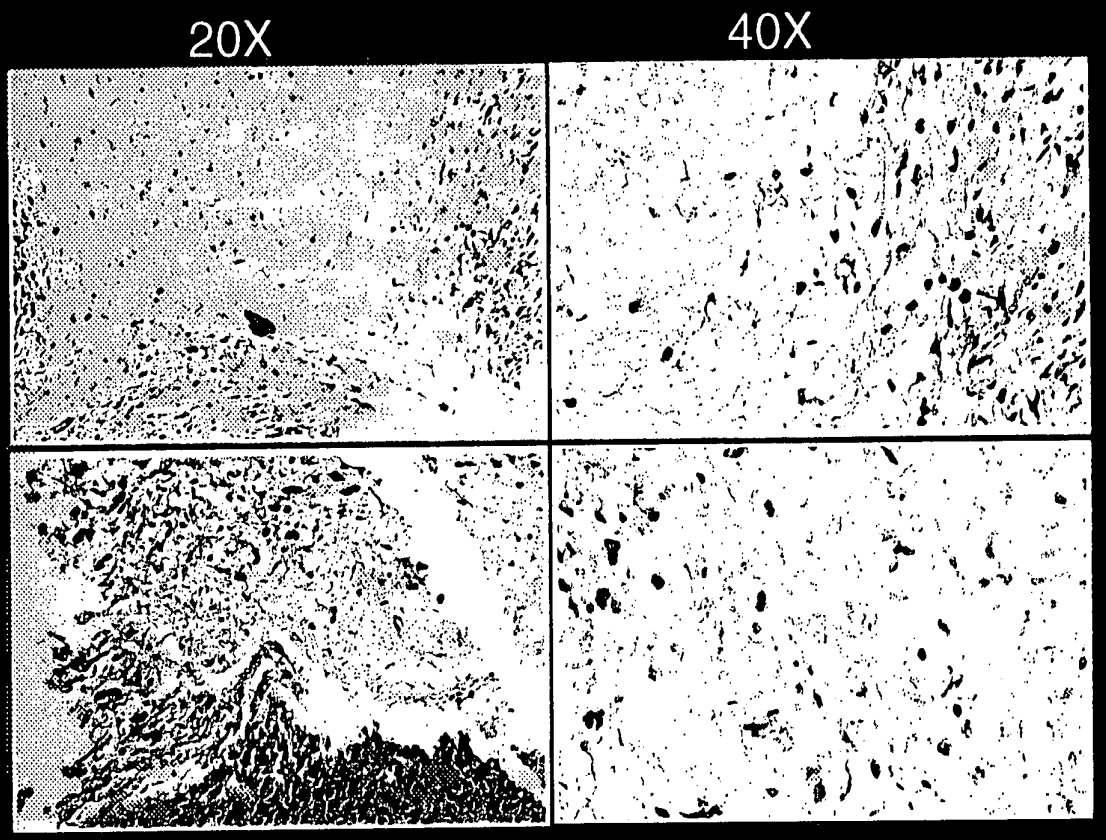


FIG 52

WO 99/50425
50/50
PCT/US99/06799
09/647418